Modulation of Scavenger Receptors by Hypoxia:

Implications for Atherosclerosis

Dissertation

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1.- Abbreviations

15-LO	15- Lipoxygenase
2-OG	2-oxoglutarate
ABCA1	ATP-binding cassette transporter A1
ABCG1	ATP-binding cassette transporter G1
acLDL	acetylated-low density lipoprotein
agLDL	aggregated-low density lipoprotein
ALOX 15-B	arachidonate *15*-lipoxygenase
ApoE/B	apolipoprotein E/B
ARNT	aryl hydrocarbon receptor nuclear translocator protein
ATP	adenosine triphosphate
BCLB	B-cell lymphoma 2
bHLH	basic helix-loop-helix
BNIP3	BCL2 adenovirus E1B 19 kDa protein interacting protein3
CA IX	carbonic anhydrase 9
CABG	coronary artery by pass surgery
CBP	CREB1-binding protein
CD	cluster of differentiation
CE	cholesterol ester
CETP	cholesteryl ester transfer protein
CHIP	Hsp70-interacting protein
CoA	coenzym A
CS-1	connecting segment 1

CXCL16	chemokine (C-X-C motif) ligand 16
DMOG	dimethyloxaloylglycin
EGF	epidermal growth factor
EGLN3	egl nine homolog 3
EPAS1	endothelial PAS domain-containing protein 1
ER	endoplasmic reticulum
FA	fatty acid
FACS	fluorescence activated cell scanning
FC	free cholesterol
FEEL-1	fasciclin, EGF-like, laminin-type EGF- like, and link
FIH	factor inhibiting HIF-1
FM	fluorescence microscopy
GLUT-1/3	glucose transporter 1/3
HAF	hypoxia-associated factor
HDL	high density lipoproteins
HIF	hypoxia-inducible factor
HRE	hypoxia response element
HSP-70	*70* kilodalton heat shock protein
ICAM	intercellular dhesion molecule 1
IFN-γ	interferon gamma
IK-β	inhibitor of NFκβ
IKK–β	Iκβ kinase
IL	interleukin
iNOS	inducible nitric oxide synthase
LCAT	lecithin-cholesterol acyltransferase

LDHA	lactate dehydrogenase A
LDL	low density lipoprotein
LIMP II	lysosomal membrane protein 2
Lox-1	lectin-like oxLDL receptor 1
LRP	low density lipoprotein receptor-related protein 1
LXR	liver x receptor
MARCO	macrophage receptor with collagenous structure
MCP-1	monocyte chemoattractant protein-1
MCSF	macrophage colony stimulating factor
MCT 4	monocarboxylate transporter 4
MHC	major histocompatibility complex
MIF	migration inhibitory factor
mmLDL	minimally modified LDL
MMP	matrix metalloproteinase
NFκB	nuclear factor kappa of activated B-cells
OCT	octamer binding transcription factor
ODD	oxygen-dependent degradation
OLR1	oxidized low-density lipoprotein receptor 1
ORO	oil red O
ORP 150	150 kDa oxygen-regulated protein
oxLDL	oxidized low density lipoprotein
p300/CREB	p300 cAMP response element-binding protein
PAS	Per-ARNT-Sim
PDGF	platelet derived growth factor
PFK	phosphofructokinase

PHD	prolyl hydroxylase domain-containing protein
PPAR	peroxisome proliferator-activated receptors
RACK 1	receptor for activated protein kinase C 1
ROS	reactive oxygen species
SCAP	SREBP cleavage-activating protein
SCARA5	class-A scavenger receptor *5*
SIRT-1	sirtuin-1
SRA	scavenger receptor A
SRB	scavenger receptor B
SRC 1	steroid receptor coactivator 1
SRCL	scavenger receptor with a C-type lectin domain
SREBP	sterol regulatory element binding protein
SREC	scavenger receptors expressed by endothelial cells
SR-PSOX	SR-PSO*X/CXC* chemokine ligand 16 (CXCL16)
C-TAD	C-terminal transactivation domain
N-TAD	N-terminal transactivation domain
TNF-α	tumor necrosis factor-alpha
VCAM-1	vascular cell adhesion molecule 1
VEGF	vascular endothelial growth factor
VHL	von Hippel - Lindau
VLDL	very low density lipoprotein
VSMC	vascular smooth muscle cell

2. Zusammenfassung

Arteriosklerotische Plaques im fortgeschrittenen Stadium bilden einen hypoxischen Kern aus, aufgrund der sich verdickenden Arterienwand und der daraus resultierenden verminderten Sauerstoffdiffusion. Cholesterinhaltige Makrophagen (schaumzellen) spielen eine Schlüsselrolle in der Ausbildung dieser Plaques, denn sie exprimieren sog. „Scavenger Rezeptoren“, die an der Aufnahme von Lipiden beteiligt sind. Die unterschiedlichen Expressionsmuster dieser Rezeptoren unter hypoxischen Bedingungen müssen jedoch noch untersucht werden. Mit Hilfe von qPCR, Zytometrie und Proteinanalyse mittels Western Blot fanden wir erhöhte mRNA und Proteinexpressionslevel des „Scavenger Rezeptors A“ (SRA) und des „Clusters of Differentitation 36“ (CD36) unter Zugabe des oxidierten Lipoproteins niedriger Dichte (oxLDL). Jedoch waren die Expressionslevel beider Rezeptoren vermindert, wenn Makrophagen Hypoxie ausgesetzt wurden. Im Gegensatz dazu war der mRNA und Proteinexpressionspegel des „ Scavenger Receptors Lektin-Like oxLDL“ (Lox-1) in Hypoxie erhöht. Mit Hilfe der Zytometrie konnte der erhöhte intrazelluläre Lipidgehalt in hypoxischen Makrophagen bestätigt werden. Des Weiteren konnte mit Hilfe einer Blockade des Lox-1 Rezeptors ein reduzierter Lidpidgehalt und eine verminderte Aufnahme von oxidierten LDL in Makrophagen beobachtet werden. Immunohistochemische Analysen menschlicher Koronarplaques zeigten eine Reduktion des SRA und des CD36 Rezeptors entlang eines Gradienten von der Adventitia zur Intimia hin, während Lox-1 hauptsächlich im hypoxischen Kern des Plaques nachweisbar war. Die Expressionsreduktion des Hypoxie induzierbaren Faktors 1α (HIF-1α) unter Verwendung der siRNA und shRNA Methode änderte die Expressionspegel der SRA und Lox-1 Rezeptoren in hypoxischen Makrophagen. Diese Ergebnisse demonstrieren, dass Hypoxie die Expression der oxLDL Rezeptoren unterschiedlich reguliert und dass Lox-1 die Hauptrolle bei der Hypoxieabhängigen Bildung von cholesterinhaltigen Makrophagen spielt.

3. Summary

Advanced atherosclerotic plaques become hypoxic in their core as the arterial wall thickens and oxygen diffusion capacity is impaired. Macrophage-derived foam cells in atherosclerotic lesions play a pivotal role in plaque formation by expressing scavenger receptors that are involved in lipid uptake. Their differential expression in the context of hypoxia remains to be determined. Using qPCR, flow cytometry and Western blotting approaches, we found that mRNA and protein expression levels of the scavenger receptor A (SRA) and the cluster of differentiation 36 (CD36) were upregulated by oxidized low-density lipoprotein (oxLDL), but decreased following exposure of macrophages to hypoxia. In contrast, scavenger receptor lectin-like oxLDL 1 (Lox-1) mRNA and protein levels were upregulated under hypoxic conditions. Flow cytometry confirmed the increased lipid content in macrophages after exposure to 0.2% oxygen (hypoxia). Furthermore, blockage of Lox-1 decreased the hypoxic induction of lipid content and oxLDL uptake in macrophages. Immunohistochemistry of human coronary plaques showed a gradual decrease of SRA and CD36 from the adventitia to the intima, while Lox-1 was mainly found in the inner core of the plaque, where an increased tissue hypoxia is present. shRNA or RNAi mediated knock-down of hypoxia-inducible factor (HIF)-1α in macrophages altered the expression of SRA and Lox-1 receptors in hypoxia. Our results demonstrate that hypoxia differentially regulates the expression of the three oxLDL receptors and that Lox-1 plays a major role in hypoxia-induced foam cell formation.

4. Introduction

4.1 Atherosclerosis

Atherosclerosis, the main cause of myocardial infarction and stroke, is a chronic and progressive inflammatory disease. It refers to the development of an atheroma plaque in the inner lining of the arteries, a normally small area between the endothelium and the underlying smooth muscle cells of the media, and results from the interaction between modified low-density lipoproteins (LDL), activated endothelial cells, monocyte-derived macrophages, T-cells, and the vessel wall [1].

Diverse genetic and environmental risk factors have been identified by epidemiological studies, such as hypertension, diabetes, insulin resistance, obesity, environmental factors, smoking, lack of exercise, high fat diet, amongst others. But the elevated levels of serum cholesterol are probably unique in being sufficient to drive the development of atherosclerosis in humans and experimental animals, even when other known risk factors are absent. The investigation of molecular mechanisms that control cholesterol biosynthesis and serum cholesterol levels led to the development of statins [2], a potent class of cholesterol lowering drugs that reduce cardiovascular mortality in hypercholesterolemic patients. However, available statins are not sufficient to fully prevent the progression of the disease in susceptible individuals.

An atherosclerotic lesion starts in the form of a fatty streak underlying the endothelium of large arteries. The principal cellular events that contribute to its formation are the recruitment of macrophages and its subsequent uptake of LDL. Several lines of evidence have already shown that modifications in the lipid component and the apolipoprotein B (apoB) parts of LDL drive the initial steps in the formation of a fatty streak [3].

The stability of atherosclerotic plaques is given by the extracellular matrix and a thick fibrous cap. An unstable plaque has a thin fibrous cap and thrombus at the

shoulder, it includes many inflammatory cells and a large lipid core. The plaque ruptures when the fibrous cap breaks and the necrotic lipid core (which is very thrombogenic) is exposed to the blood in the arterial lumen. The margins of the plaque contain macrophages, T lymphocytes and a paucity of smooth muscle cells. Fissures and plaque rupture are the cause for the great majority of thrombi that lead to acute coronary syndromes [4]. Figure 1 depicts the evolution of atherogenesis, from an initial stage up to the ruptured plaque and formation of a thrombus.

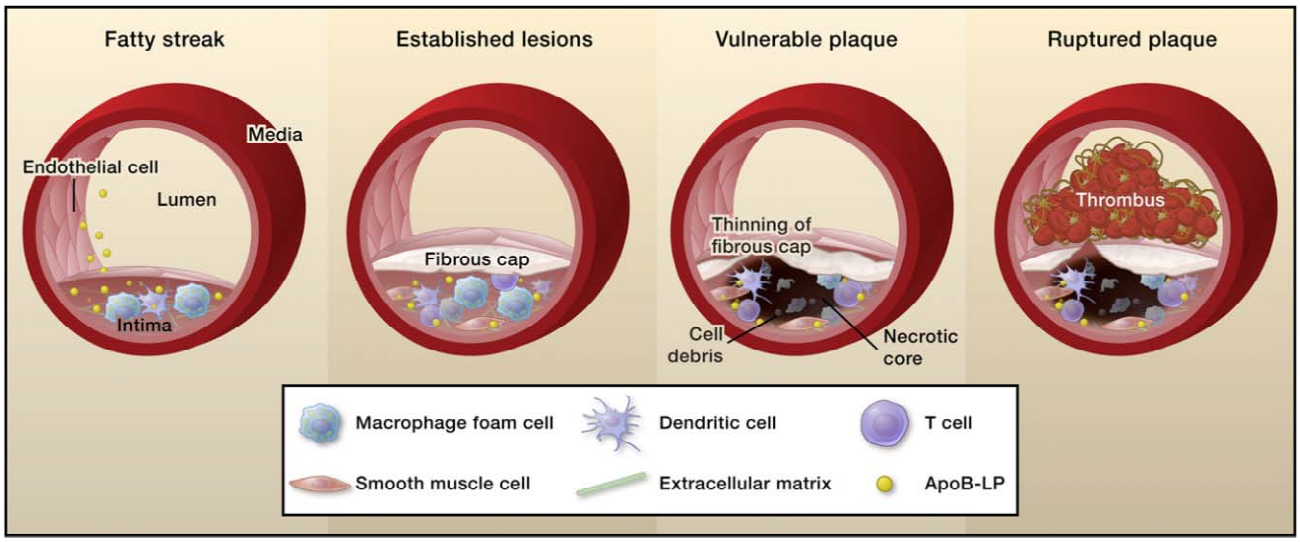

Fig. 1 Progression of an atherosclerotic lesion: from a fatty streak to the ruptured plaque (from [5]).

Infiltration of macrophages into the subendothelial space is a key step in early atherogenesis. Eventually, these inflammatory macrophages ingest oxLDL via scavenger receptors and become foam cells, thereby promoting plaque formation.

4.1.1. Initiating events: LDL modification and monocyte/macrophage recruitment

Under normal conditions LDL is protected from oxidation in the plasma compartment, but when retained by extracellular matrix proteins in the artery wall, it gets susceptible to enzymatic and non-enzymatic modifications. Studies tried to understand potential oxidant-generating systems that could directly or indirectly target LDL. Some of them include mielo peroxidase, NADPH oxidase, nitric oxide synthase and 15-lipoxygenase (15-LO) [3].

In spite that monocyte and macrophage recruitment to the arterial wall can have an initial protective function, it can ultimately lead to the development of the atherosclerotic lesion [6]. Macrophages initially remove cytotoxic and proinflammatory oxLDL particles or apoptotic cells, but progressive accumulation of macrophages and their accumulative uptake of oxLDL make them become a foam cell, which is a hallmark for the progression of the disease.

As mentioned above, LDL is exposed to oxidative modifications in the subendothelial space, progressing from minimally modified LDL (mmLDL), to extensively oxidized LDL (oxLDL). Circulating monocytes attach to the endothelial layer of cells that have been induced to express cell adhesion molecules. Once attached to the endothelial cell, monocytes enter the intima by diapedesis between the endothelial cells and their junctions. Families of chemoattractant cytokines (chemokines) have been defined as capable of recruiting macrophages into the arterial intima. It has been shown that, for example, overexpression of monocyte chemoattractant protein-1 (MCP-1) can recruit monocytes that characteristically accumulate in the early lesion. IL-8, IFN-γ and other chemokines may have similar function as MCP-1 during atherogenesis [6].

The recruitment of monocytes to the sites that are more prone to develop an atherosclerotic lesion in the arteries, is regulated by cell adhesion molecules that are expressed on the surface of endothelial cells in response to inflammation. Several

cell adhesion molecules have been identified to play roles in macrophage recruitment. One of the first to be implicated was VCAM-1, based on its increased expression on endothelial cells over lesion-prone areas [7]. Furthermore, E selectin and P selectin play significant roles in monocyte entry based on a 40% to 60% decrease in atherosclerosis in apo E–deficient mice that lack genes which codify for both selectins [8]. Moreover, gene deletion of ICAM-1 resulted in the reduction of monocyte recruitment to atherosclerotic lesions in apo E–deficient mice [9] . Another study with a peptidomimetic that corresponds to the connecting segment 1 (CS-1) domain of fibrinectin 1 (which blocks the function of adhesion molecule VLA-4 on the leukocyte surface) decreased lipid accumulation in C57BL/6J mice fed an atherogenic diet [10]. Taking together, these findings suggest that there are many cell adhesion molecules involved in the recruitment of monocytes and T cells to the atherosclerotic lesion. Neutrophils, which normally contribute to most inflammatory responses, are interestingly absent in lesions, and the mechanisms leading to their absence remain to be defined. Figure 2 depicts the initiating events in the development of a fatty streak.

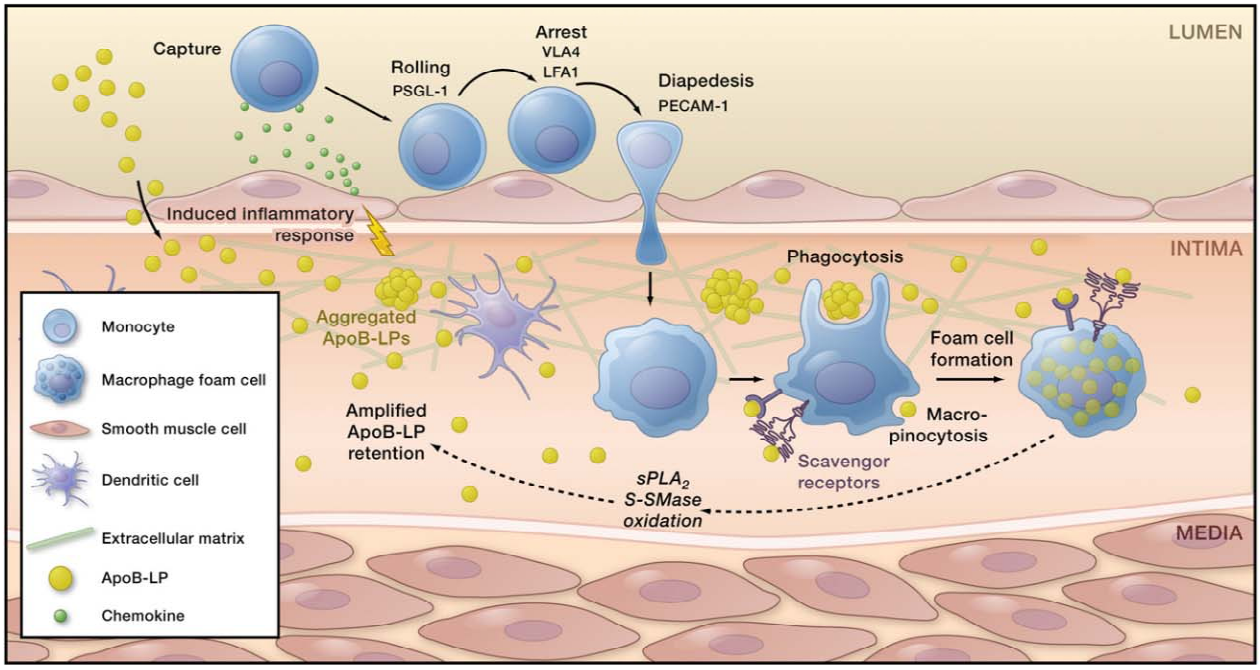

Fig. 2 Initiating events in the development of a fatty streak (from [5])

When monocytes adhere to the endothelial layer, they migrate into the subendothelial space and get exposed to cytokines and factors, such as the Macrophage colony stimulating factor (MCSF) which leads to macrophage differentiation. When monocytes differentiate into macrophages, they upregulate a set of proteins, including the so called scavenger receptors. Uptake of modified LDL via scavenger receptors leads to foam cell formation [11]. OxLDL cholesterol taken up by scavenger receptors, such as scavenger receptor A (SRA), cluster of differentiation 36 (CD36) or lectin-like oxLDL receptor 1 (Lox-1), amongst others, is subject to esterification and storage in lipid droplets.

4.1.2 Foam cell formation: balance between cholesterol uptake and efflux

An important and characteristic hallmark of both early and advanced atherosclerotic lesions is the development of macrophage “foam cells” that contain massive amounts of cholesterol esters in the form of lipid droplets. Cholesterol accumulation in these cells is due to the uptake of modified forms of LDL through the above mentioned scavenger receptors. The recognition of oxLDL by the scavenger receptors is through their apo B part [12]. It has been demonstrated that amongst all the scavenger receptors involved in lipid uptake, SRA, CD36 and Lox-1 play significant roles. Apo E-deficient mice lacking either of these three receptors developed less atherosclerosis than control apo-E deficient mice [13].

Macrophage normally dispose the excess of cholesterol mainly through membrane transporters, with HDL serving as the principal extracellular acceptor. In vitro studies have broadly indicated that ABC A1 mediates transport of cholesterol from cells to HDL acceptors. HDL is esterified to cholesterol esters by lecithin-cholesterol acyltransferase (LCAT), and it can subsequently exchange cholesterol esters for triglycerides carried by other lipoproteins via cholesterol ester transfer protein (CETP). Furthermore, HDL can also deliver cholesterol esters to the liver for excretion by binding to the scavenger receptor SR-B, which also binds HDL. The physiologic importance of SR-B for reverse cholesterol transport is suggested by the findings that hypercholesterolemic mice homozygous for a hypomorphic SR-B1 allele

develop increased atherosclerosis, while mice that overexpress SR-B1 exhibit reduced atherosclerosis [14] [15]. Figure 3 depicts foam cell formation and the balance between lipid uptake and efflux.

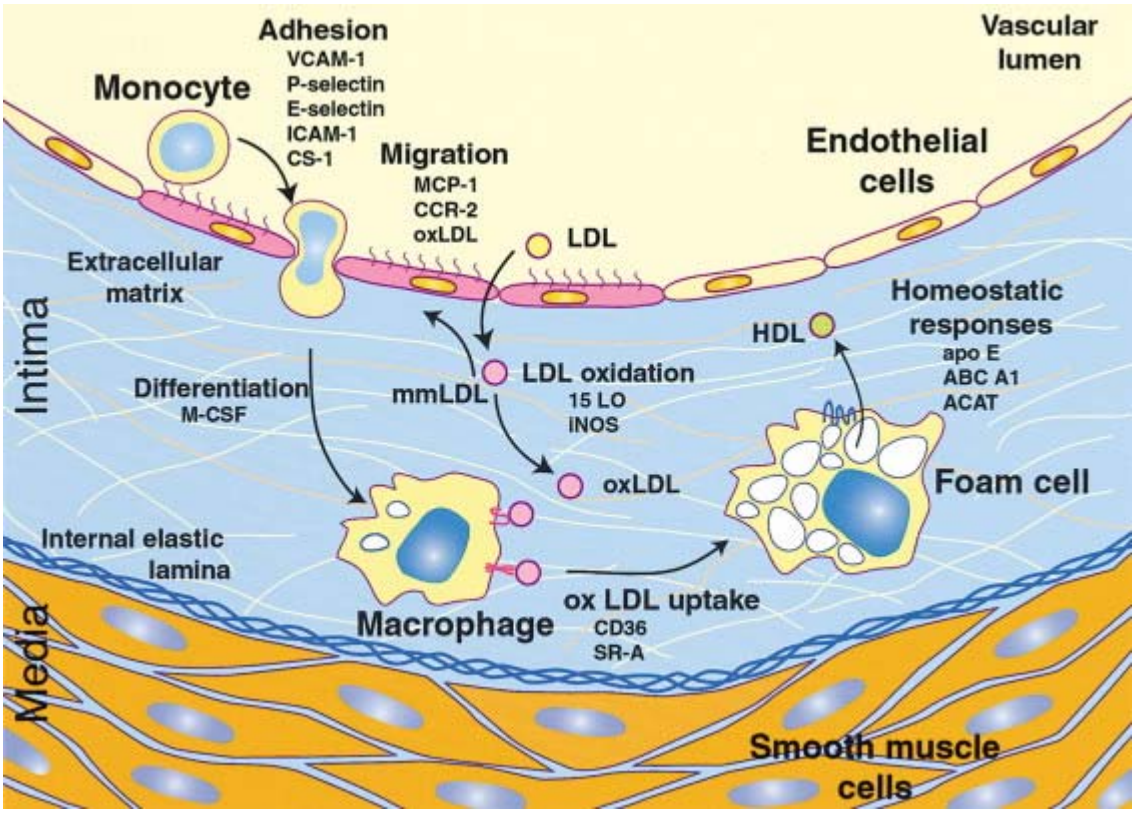

Fig. 3 Foam cell formation: balance between lipid uptake and efflux (from [3]) .

The atherosclerotic lesion progresses when interactions between macrophage foam cells, Th1 and Th2 cells establish a chronic inflammatory process. Cytokines secreted by lymphocytes and macrophages exert both pro- and antiatherogenic effects on each of the cellular elements of the vessel wall. Afterwards, smooth muscle cells migrate from the medial portion of the arterial wall, they proliferate and secrete extracellular matrix proteins which form a fibrous plaque.

4.1.3 Inflammation, atheroma progression and complication.

When the fatty streak is formed, the early lesion evolves to an advanced and complex one which eventually leads to a ruptured stage. Smooth muscle cells multiply, accumulate in the plaque and excrete extracellular matrix. As the lesion starts growing more and more, the arterial lumen narrows until hinders blood flow and leads to clinical manifestations; either unstable *angina pectoris*, in the coronary circulation, or an acute coronary infarction.

This phase of lesion development is characterized by interactions between monocyte/macrophages and T cells that result in a broad range of cellular and immunological responses and the acquisition of many features of a chronic inflammatory state. Cross talk appears to occur among the cellular elements of developing lesions. Lesional T cells appear to be activated, expressing both Th1 and Th2 cytokines [16]. Similarly, macrophages, endothelial cells, and smooth muscle cells get activated and express MHC class II molecules and numerous inflammatory products, such as TNFα, IL-6, and MCP-1. Figure 4 depicts lesion progression.

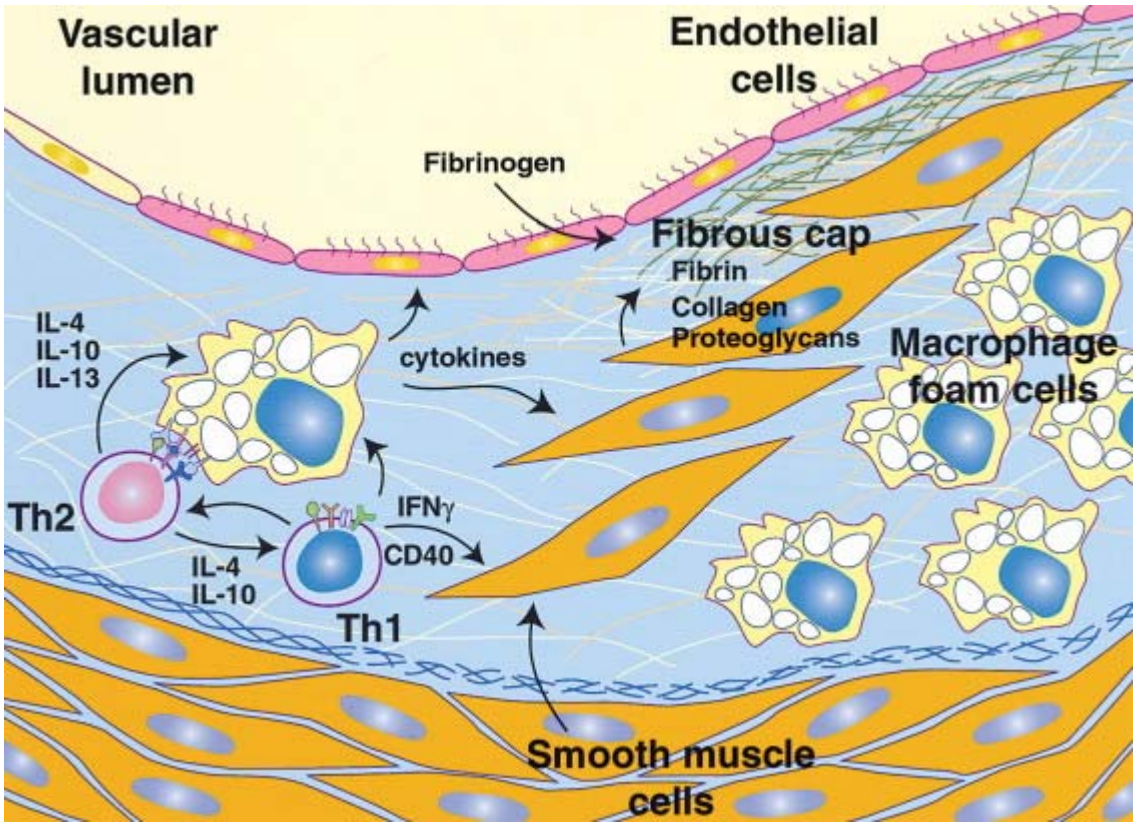

Fig. 4 Lesion progression (from [3])

The immune responses involved can have both atherogenic and antiatherogenic properties. Single cytokines can produce both positive and negative effects. Interferon γ (IFNγ), a Th1 cytokine, can reduce scavenger receptor expression on macrophages, decreases collagen synthesis, and inhibit smooth muscle cell proliferation. However, IFNγ also stimulates macrophage production of proinflammatory cytokines, increases expression of MHC class 2 molecules and accumulation of macrophages within lesions. In mice, IFNγ is considered to be atherogenic, as apo E–deficient mice lacking the IFNγ receptor showed less atherosclerosis than control apo E–deficient mice [17].

Th2-derived cytokines also have an important influence on the atherosclerotic lesion. Interleukin-4 (IL-4) can be antiatherogenic by antagonizing INFγ activity in macrophages and inhibiting Th1 cell function. However, IL-4 induces 15-LO, which promotes LDL oxidation and hence atherosclerosis. IL-10 has deactivating properties in macrophages, modulating processes that may interfere with the development and

stability of the atherosclerotic plaque. IL-10-deficient mice exhibit an increase in lipid accumulation, suggesting the role of IL-10 in reducing foam cell formation [18].

4.1.4 Advanced lesions and plaque disruption

Macrophages contribute to formation of the necrotic core and thinning of the fibrous cap, which characterizes the vulnerable plaque. Thrombosis in the lumen needs communication between procoagulant and prothrombotic factors in the intima with platelets and coagulation factors in the lumen [5]. Matrix metalloproteinases derived from macrophages contribute to fibrous cap thinning. Macrophage-derived serine proteases also contribute to the degradation of collagen and elastin in advanced lesions [19]. Figure 5 illustrates a advanced lesion and instability of an atherosclerotic plaque. Physical disruption of the atherosclerotic plaque may trigger thrombosis and promote the expansion of the lesion.

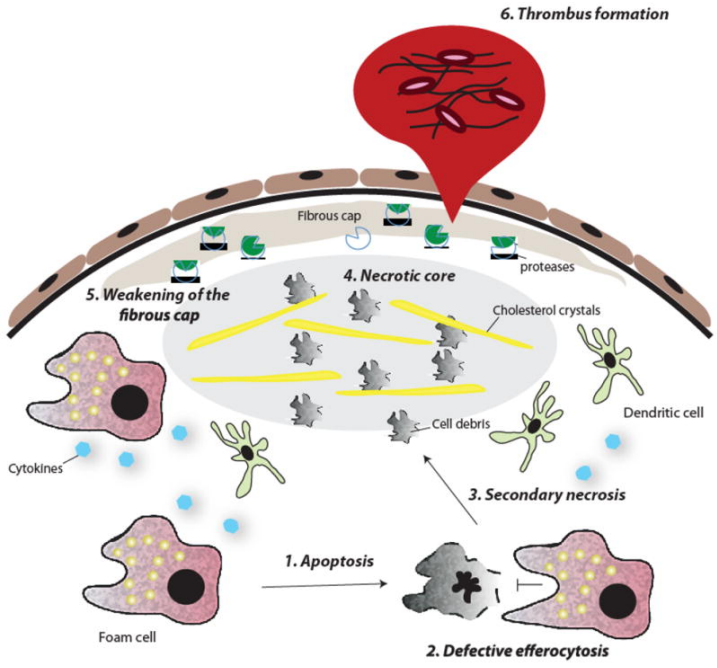

Fig. 5 Advanced lesion and instability of the atherosclerotic plaque (from [5]

Macrophage apoptosis and ineffective clearance in advanced lesions leads to the formation of a necrotic core. Death of smooth muscle cells and protease degradation of extracellular matrix weakens the fibrous cap, making it more prone to rupture. Exposure of the thrombogenic material in the lesion causes platelet aggregation and the formation of thrombi. Macrophages contribute with the overall process secreting cytokines, proteases and thrombogenic factors. Myocardial infarctions and strokes are generally resulting from plaque rupture and thrombosis, narrowing of the vessels in advanced atherosclerotic plaque can lead to isquemia [20]. Furthermore, angiogenesis occurs in association with protease activation in surrounding tissues, and it is suggested that neovascularization could contribute to plaque instability and rupture [21].

4.2 Foam cell formation: lipid uptake and efflux

As described before, macrophages loaded with lipid droplets result in cells with a foamy appearance becoming the so-called “foam cells” and accumulation of their lipids is mediated through scavenger receptors. Release of foam cell contents trigger the migration of more macrophages into the intima of the vessel, initiating a vicious cycle in the formation of a necrotic core consisting of cell debris, lipids and cholesterol crystals.

Since more than a decade ago, the formation of foam cells has been recognized to be important for atherosclerotic development but its mechanism has only been studied until recently [22]. It is believed that there are different subsets of macrophages which play different roles in atherogenesis. Subsets of macrophages are classified according to their surface markers and chemokine receptors, and there are mainly two groups: M1 or classically activated macrophages, and M2 or alternatively activated macrophages. M1 macrophages, or classically activated macrophages, are characterized generally by interleukin (IL)–12^{high}, IL-23^{high}, IL-10^{low} phenotype. M1 macrophages produce high amounts of reactive oxygen and nitrogen intermediates as well as inflammatory cytokines. Classically activated macrophages are involved in the polarized Th1 responses and mediate resistance against intracellular parasites and tumors [23].

The M2 type show high levels of scavenger, galactose-like and mannose receptors in opposition to M1 or classically activated macrophages. M2 have an increased ability to phagocyte tissue debris and prevent tissue damage, compared to the other population [24]. Recent studies showed another macrophage subtype, Mox, which is found in the oxLDL-rich microenvironment of atherosclerosis. Mox macrophages have lower chemotactic and phagocytic capacities. However, these studies have been conducted *in vitro* only, because of the complexity of atherosclerotic lesions regarding heterogeneity of macrophages [25].

Macrophages take up extracellular modified lipoproteins through receptor-phagocytosis and pinocytosis. Since four years it is believed that the LDL receptor plays a major role in this process [26], but foam cells are even found in familiar hypercholesterolemia patients with genetically impaired LDL receptors. Deficiency of ApoE and CD36 show reduced atherosclerotic lesions compared to their littermate controls (ApoE–null mice) demonstrating the importance of scavenger receptors [27]. In contrast to LDL receptors, which are known to be down regulated in conditions of increased cholesterol, scavenger receptors are not affected leading to continuous internalization of lipoproteins which accumulate in the form of lipid droplets in the cytoplasm.

When internalized, lipoproteins are delivered to lysosomes where cholesterol esters (CE) get hydrolyzed into fatty acids (FA) and free cholesterol (FC). In lysosomes acid lipases hydrolyze CEs and generate FC for the efflux through ABC transporters. On the other hand triglycerides are hydrolyzed into glycerol and fatty acids which are then esterified again and stored into lipid droplets [24].

Receptor-mediated free cholesterol efflux is another important mechanism for prevention of foam cell formation. Passive diffusion plays a minor role, but active transport via several transporters is responsible for the efflux of cholesterol from macrophages by ABCA1 and ABCG1 [28]. Besides ABC transporters, also the scavenger receptor B (SRB) is atheroprotective because it is involved in cholesterol

efflux. Genetically suppressed SRB in ApoE-deficient mice accelerates dramatically the progression of the disease [29].

Moreover, cellular lipid homeostasis is regulated through transcription factors, such as sterol regulatory element-binding proteins (SREBPs) and peroxisome proliferator-activated receptors (PPARs). SREBPs play a main role in keeping lipid homeostasis and regulate many genes involved in cholesterol homeostasis and fatty acid biosynthesis [30] .

4.3 Scavenger receptors

Scavenger receptors are structurally diverse proteins. When originally identified, by Brown and Goldstein in 1977, they were defined by their capacity of binding modified forms of low density lipoproteins, including oxidized LDL (oxLDL) and acetylated LDL (acLDL) and considered as pro-atherogenic [2]. While native LDL is removed from the circulation by the LDL receptor, modified LDL cannot use this pathway and binds to scavenger receptors instead, leading to uncontrolled lipid overload and foam cell formation [31].

According to the classification done by Krieger and colleagues [32], the family of scavenger receptors includes eight different classes of membrane and soluble proteins encoded by different and unrelated genes. Figure 6 shows the members of the scavenger receptor family, which are grouped based on their structural domains, rather than sequence similarity[33].

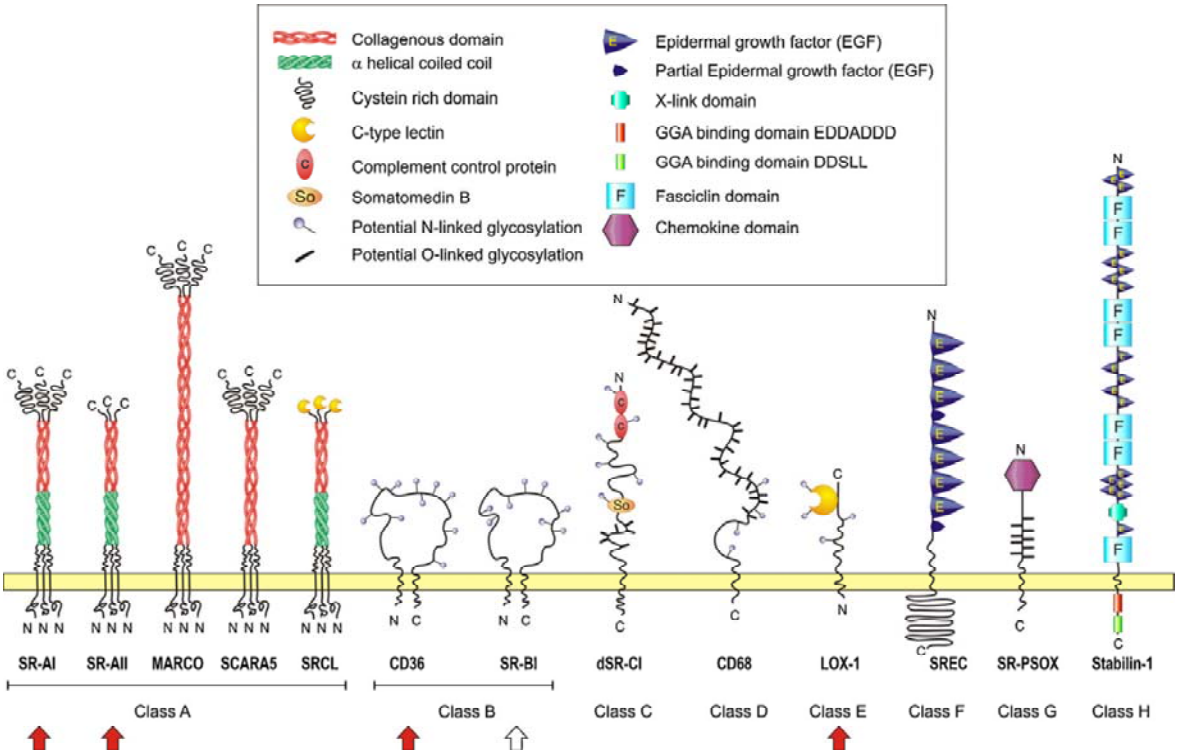

Fig. 6 Members of the scavenger receptor family (from [31].

Most of the 8 classes of scavenger receptors are implicated in atherogenesis, contributing to foam cell formation by interacting with oxLDL or by additional mechanisms as described below. Scavenger receptors perform clearance of unwanted macromolecules, pathogens and specially modified lipoproteins, using receptor mediated endocytosis. In general, endocytic receptors recognize extracellular ligands, and then invaginates them forming clathrin complexes and vesicles. Vesicles contain complexes of receptor-ligand and are delivered to endosomes. Scavenger receptors can then dissociate from their ligands and recycle to the surface of the cell where they internalize new portions of the ligand. In some cases, scavenger receptors remain associated with their ligands and are degraded in lysosomes [31].

Class A scavenger receptors include four related genes: scavenger receptor A (SR-A), macrophage receptor with collagenous structure (MARCO), scavenger receptor with C-type lectin (SRCL) and scavenger receptor A-5 (SCARA5) [33]. The *SR-A* genes

produce three (SR-AI/II/III) splice variants. SR-AI and II are largely found on macrophages but they can also be present on endothelial cells and vascular smooth muscle cells. SR-A is postulated to be proatherogenic because of its capacity to bind oxLDL contribuing to foam cell formation.

Class B scavenger receptors contain four members: CD36, SR-B, LIMPII-related genes and CD163. *CD36* expression includes cells of lymphoid and hematopoietic lineages including leukocytes, platelets, endothelial cells, adipocytes, VSMCs and some epithelial cells; its levels are highest in macrophages [33].

Class C receptors only includes dSR-C1 which has been so far only found in *Drosophila melanogaster*. dSR-C1 is a pattern receptor for bacteria present in hemocytes and macrophages during the embryonic development of the fly. **Class D** includes CD68 and Lamp, a lysosomal membrane glycoprotein. **Class E** consists of only one member: the lectin-like oxidized low density lipoprotein receptor 1 (Lox-1). *Lox-1* (OLR1) is expressed on endothelial cells, macrophages, smooth muscle cells and platelets. Its resting levels are low but they are increased by proinflammatory stimuli including oxLDL, inflammatory cytokines, TNF-*α*, shear stress, oxidative stress, phorbol ester, endothelin-1 and angiotensin II. Lox-1 activates foam cell formation and the incidence of atherosclerotic plaques is significantly lowered in Lox-1-deficient mice. **Class F** consists of the SREC, a scavenger receptor expressed by endothelial cells and macrophages. The chemokine ligand CXCL16 is a **class G** scavenger receptor. It binds phosphatidylserine and oxidized lipoprotein (SR-PSOX). Its expression is increased by TNF-*α*, IFN-*γ*, LPS and oxLDL stimulation. **Class H** scavenger receptors includes fasciclin, EGF-like, lamin type EGF-like and link domain-containing scavenger receptor-1 (FEEL-1) [33].

The best investigated scavenger receptors involved in foam cell formation are CD36 and SRA. Kunjathoor and coworkers generated mice lacking both SR-A and CD36 to investigate their combined impact on macrophage lipid uptake and verify the contribution of other SRs to this process. They demonstrated that SR-A and CD36 account for 75-90% of degradation of LDL modified by acetylation or oxidation.

Cholesteryl ester derived from modified lipoproteins did not accumulate in macrophages taken from SRA-CD36 deficient mice. With these results they suggested that SR-A and CD36 are responsible for the principal modified LDL uptake in macrophages and that other scavenger receptors do not have a relevant compensation when they are absent [34].

Kunjathoor demonstrated that both, SRA and CD36, are the main receptors responsible for foam cell formation. However, Moore and coworkers showed on the other hand, aortic sinus lesions with abundant foam cells in SRA and CD36 knockout mice [5]. This finding indicates the relevance of other scavenger receptors in foam cell formation as well.

Lox-1 overexpression in transgenic mouse models has been recently studied to explore its functional role in atherosclerosis. Inaoe observed that ApoE mice with elevated Lox-1 levels displayed accelerated intramyocardial vasculopathy. They showed that the increase of Lox-1 in coronary vessels and cardiomyocytes correlated with enhanced oxLDL uptake [35]. *In vitro* studies have shown that oxLDL-mediated activation triggers an endothelial Lox-1-linked signaling pathway, leading to pro-inflammatory responses. Lox-1 activation stimulates ROS production and activates nuclear factor κB (NF-κB) which translocates into the nucleus results and activates transcription of genes encoding pro-inflammatory and adhesion molecules [36]. Lox-1 is transcriptionally upregulated by TNFα, angiotensin II, shear stress and ox-LDL [37]. Expression of Lox-1 leads to expression of adhesion molecules, CD40/40 L in endothelial cells and upregulation of matrix metalloproteinases [38]. A deeper knowledge about the regulation of this receptor and its signal transduction pathways may lead to new therapies for pathologies which involve endothelial dysfunction, such as atherosclerosis.

4.4 Atherosclerosis and hypoxia

During atherogenesis, the supply of nutrients and oxygen to the inner parts of atherosclerotic plaques likely becomes diffusion-limited. Therefore, the balance between oxygen supply and demand in the arterial wall is important for plaque progression. As the lesions grow and the number of foam cells in the intima increases, the core becomes gradually hypoxic and macrophage cellular oxygen consumption is inhibited.

Normal oxygen tension in tissue ranges from 20–100 mmHg to below 10 mmHg in normal liver and kidney [39]. *In vitro* effects of hypoxia are usually observed between 10 mmHg (1%, or 14 μM, O_2) and 1.6 mmHg (0.2% O_2). Sluimer and coworkers demonstrated the presence of hypoxia in advanced atherosclerotic lesions of symptomatic patients using pimonidazole administration prior to carotid endarterectomy and observed that hypoxia correlates with the presence of macrophages, angiogenesis and thrombus formation [40]. Oxygen measurements *in vitro* and *in situ* indicated as well that the oxygen partial pressure in the core is inversely correlated with the size of the plaque [41].

Hypoxia is present when oxygen supply is decreased and/or demand is increased [42] In the atherosclerotic lesion, oxygen diffusion may be limited due to intimal thickening or obstructed by calcifications. However, hypoxia could also occur because of the high oxygen demand of inflammatory cells, such as macrophage foam cells [43]. Hypoxia of the vessel wall has been detected when the intimal thickness exceeds the maximum oxygen diffusion distance of ~100–250 μm [40]. In rabbit atherosclerosis plaque hypoxia was observed in 200–300 μm deep regions of the rabbit plaque and specifically in macrophage-rich areas [41]. In most human lesions, a 100–250 μm hypoxia-negative rim bordered the lumen, suggesting that hypoxia resulted from plaque thickness [40].

Nevertheless, hypoxia has been shown to occur in foam cells suggesting that the inflammatory content can contribute to the presence of plaque hypoxia. Björnheden showed in human subluminal region (20–30 μm) foam cells that were already hypoxic, despite their location still within the oxygen diffusion distance [41]. As described before,

inflammation is a hallmark of the atherosclerotic plaque. Hypoxia threshold is as likely to be dependent on the inflammatory environment within the plaque. The transcription factor HIF, which will be described into more detail in the next section, plays a major role in cellular adaptation to hypoxia. It has been shown an important cross-talk between HIF hypoxia and inflammation [44].

Gao and coworkers reviewed studies which describe the role of HIF in various stages and components of atherosclerosis. Figure 7 summarizes the most important effects of HIF-1 on atherosclerosis.

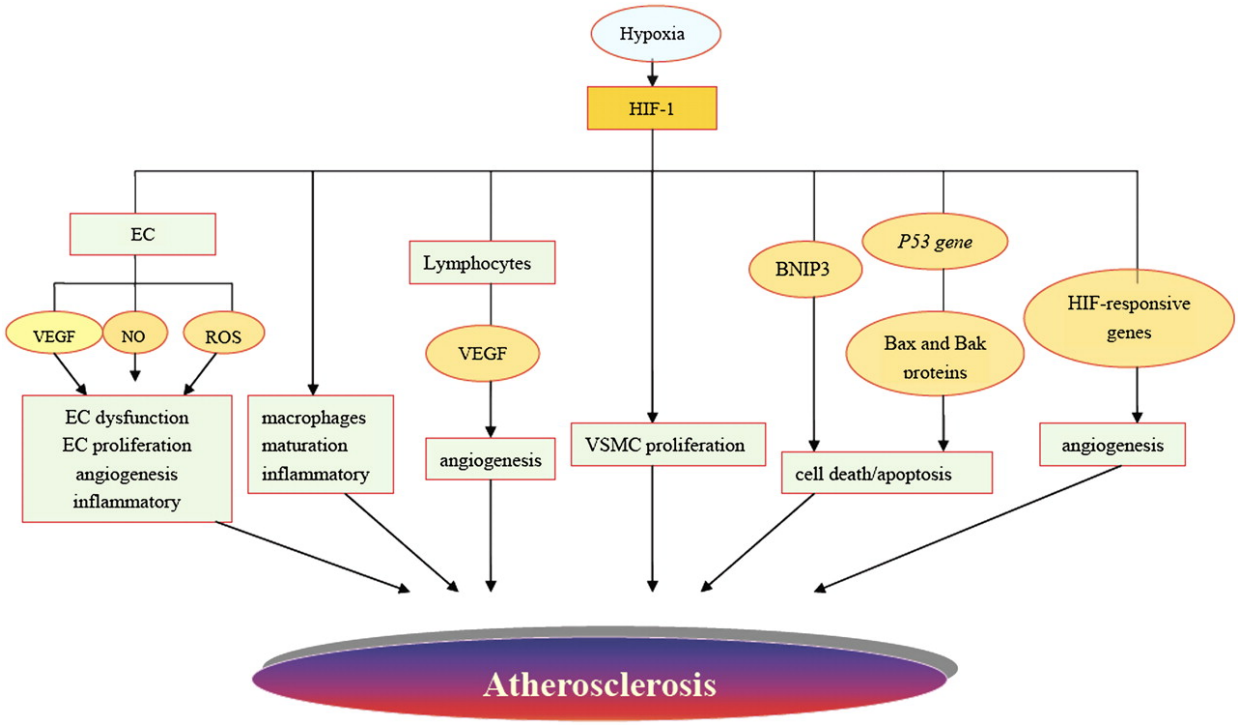

Fig. 7 Main pathophysiological effects of HIF-1 in atherosclerosis. Adapted from [45]

As it was described before, the maintenance of vascular homeostasis is performed by endothelial cells, as well as the recruitment of inflammatory cells, cytokines, and the formation of a barrier with antithrombotic functions. All of these mechanisms are perturbed in the presence of hypoxia, because of the regulatory pathways controlled by HIFs which can lead to inflammation and angiogenesis. *In vitro* studies performed by Fang et al., show stabilization of HIF-1α and HIF-2α in

macrophages experiencing hypoxia [46] and moreover, Oda and coworkers, observed that macrophage differentiation was activated by HIF-1α [47].

Hypoxia is very closely related to angiogenesis by the presence of intraplaque new vessel formation and the implication of several HIF downstream targets such as VEGF and matrix metalloproteinases. Microvessels are present in the normal adventitia, where they are called 'vasa vasorum', and in the atherosclerotic plaque. Like the vessel lumen, vasa vasorum supply oxygen to the vessel wall [48]. HIF1α co-localizes with hypoxia and macrophages [40] and is related to atherosclerosis and angiogenesis development in human coronary and carotid atherosclerosis [40] [49].

4.5 Hypoxia and cellular oxygen sensing

Hypoxia in human physiology and disease

Oxygen tensions in physiological tissue are lower than ambient oxygen tensions due to the decrease in the oxygen from the blood as it circulates from the lungs throughout the body. Koh et al [50] summarized some physiological oxygen concentrations in normal human tissue types. Table 1 describes physiological concentrations in normal human tissue samples.

Organ	Normal pO_2(mmHg)	% O_2
Trachea	150	19.7
Alveoli	110	14.5
Arterial blood	100	13.2
Pulmonary arterial blood	40	5.3
Brain	35	4.4
Intestinal tissue	58	7.6
Liver	41	5.4
Kidney	72	9.5
Muscle	29	3.8
Bone marrow	49	6.4

Table1. Physiological oxygen concentrations in a selection of normal human tissue types. From [50].

Different oxygen concentrations have major roles in human physiology. For instance, hypoxia offers the appropriate stimulus for embryogenesis, wound healing and keeps the pluripotency of stem cells. However, it can also restrict physiological functions in cells, organs and tissues as when caused by ischemia or by high altitude.

4.5.1 The hypoxia inducible factor: HIF

Semenza and Wang discovered in 1992 a nuclear factor that was induced under hypoxia in human Hep3B hepatoma nuclear extracts. They observed that this protein bound to the oxygen-responsive enhancer element of the erythropoietin gene promoting its transcription under hypoxic conditions [51]. They cloned the main players which enable aerobic organisms to adapt to hypoxia, and called them Hypoxia inducible factor (HIF).

HIFs are highly sensitive to oxygen changes and play a major role protecting the body in response to hypoxia. This is achieved through the activation of more than 200 genes, involved in cellular adaptation to hypoxia, angiogenesis and cell survival [45; 52].

HIFs are composed of one alpha and one beta subunit, which conform a heterodimer. There are three main HIF-α subunits (HIF-1α, HIF-2α or HIF-3α) and a constitutive HIF-β subunit, also known as the aryl hydrocarbon receptor nuclear translocator (ARNT), which together constitute the HIF-1, HIF-2 and HIF-3 complexes respectively [53]. The better studied are HIF-1 and HIF-2; HIF-3 shares with both a Per-Arnt-SIM (PAS) and a basic helix-loop-helix (bHLH) domains, but is lacking a C-terminal transactivation domain (C-TAD).

The bHLH and PAS domains are responsible for heterodimerization and DNA binding; on the other hand, the oxygen-dependent degradation (ODD) domain is required for oxygen-dependent hydroxylation and degradation. The N-terminal and C-terminal transactivation domains (N-TAD and C-TAD, respectively), are required for transcriptional activation.

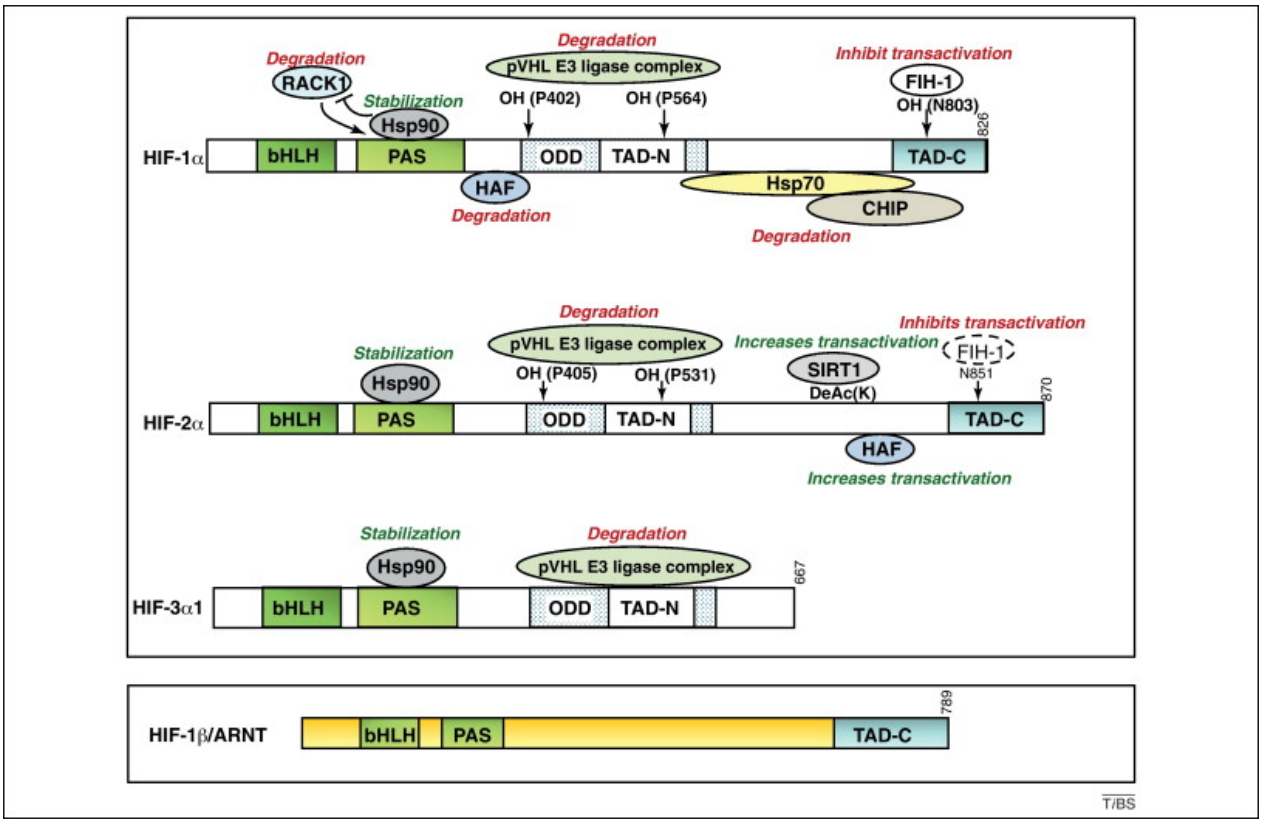

Fig. 8 The structural domains of the hypoxia-inducible factor. Adapted from [50]

Figure 8 shows the binding domains of some of the known modulators of HIF-α (red fonts indicate inhibitory interactions, green fonts indicate activating interactions). The von Hippel–Lindau protein (pVHL) E3 ligase complex is known to regulate all three HIF-α subunits. The factor inhibiting HIF (FIH) hydroxilates HIF-α subunits [50]. Figure 8 further depicts that hypoxia-associated factor (HAF) selectively binds to HIF-1α and HIF-2α, leading to degradation and transactivation, respectively. Hsp70 enhances the binding to the carboxy terminus of Hsp70-interacting protein (CHIP) to HIF-1α, resulting in HIF-1α degradation. Sirtuin 1 (SIRT1) participates in deacetylation of HIF-2α, resulting in its activation [50].

In 1996, Jiang and coworkers observed in HeLa cells that HIF-1α levels increase exponentially in low oxygen concentrations with a maximal response at 1 to 0.5% O_2

and maximal HIF levels induced at concentrations below 0.5% O_2 [54]. Under normoxic oxygen concentrations, HIFα subunits are hydroxylated by prolyl hydroxylase domain containing (PHD) enzymes which target HIFα for polyubiquitinilation by the von Hippel-Lindau protein (VHL) and proteosomal degradation. However, when oxygen is insufficiently available, PHDs are inhibited. This leads to an accumulation of HIFα subunits, which translocate into the nucleus, bind to highly conserved hypoxia responsive elements (HREs) and activate downstream targets for cellular adaptation to hypoxia. Figure 9 illustrates HIF regulation by oxygen.

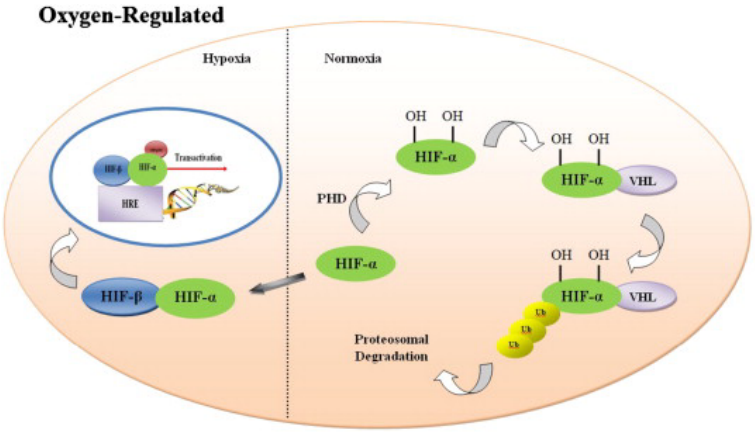

Fig. 9 HIF regulation by oxygen. Adapted from [55]

Under aerobic conditions, HIF-□ subunits are hydroxylated by PHDs at two conserved proline residues (P402/P564 and P405/P531 for human HIF-1α and HIF-2α, respectively) situated within the ODD domain. Hydroxylation requires oxygen, 2-oxoglutarate (2-OG), ascorbate, and iron (Fe^{2+}) as cofactors. The hydroxylation of HIF□ subunits facilitates the binding of pVHL to the HIF-1/2α ODD [56]. This in turn forms the substrate recognition module of an E3 ubiquitin ligase complex comprising elongin C, elongin B, cullin-2, and ring-box 1, which directs HIF-1/2α polyubiquitylation and proteasomal degradation [57].

Under hypoxic conditions, PHD activity is inhibited, pVHL binding is blunted, and HIF-1α and HIF-2α accumulate and enter the nucleus where they heterodimerize with

HIF-β and bind to the HRE to transactivate a variety of hypoxia-responsive genes. As mentioned above, in normoxia the ability of HIFs to activate transcription is further prevented by another oxygen-regulated enzyme, factor inhibiting HIF-1 (FIH-1). FIH-1 hydroxylates Asn 803 within the TAD-C of human HIF-1α, disrupting its interaction with the transcriptional co-activators p300/CREB-binding protein (CBP) [58]. As with the PHDs, Asn hydroxylation is inhibited under hypoxic conditions, allowing the p300/CBP complex to bind to HIF-1/2α, allowing HIF transactivation.

4.5.2 HIF-1 versus HIF-2 activation

Since its identification over a decade ago, HIF-1α has been described as the master regulator of the hypoxic response. HIF-2α was initially identified as the endothelial PAS domain protein (EPAS1), an endothelium-specific HIF-α isoform, and was therefore considered to have a more specialized function than HIF-1α [59]. However, HIF-2α is also expressed in brain, heart, lung, kidney, liver, pancreas, and intestine, suggesting that it also has a major role in the hypoxia response.

Now it is well known that both HIF-1α and HIF-2α participate in hypoxia-dependent gene regulation. Although both HIF-1 and HIF-2 bind to the same HRE consensus sequence in the regulatory regions of target genes, DNA binding does not necessarily correspond to increased transcriptional activity, suggesting that post-DNA binding mechanisms might be required for transactivation [60].

In general, HIF-1 preferentially induces genes that encode for glycolytic enzymes, such as phosphofructokinase (*PFK)* and lactate dehydrogenase A (*LDHA)*; those involved in pH regulation, such as monocarboxylate transporter 4 (*MCT4*) and carbonic anhydrase 9 (*CA-IX*); and those that promote apoptosis, such as BCL2/adenovirus E1B 19kDa interacting protein 3 (*BNIP3*) and BCL2/adenovirus E1B 19kDa interacting protein 3-like (*BNIP3L/NIX*) [50].

On the other hand, HIF-2 induces genes that are involved in invasion, including the matrix metalloproteinases (*MMP*) *2*, and *13*, and the stem cell factor *OCT-3/4 [61]*. However, HIF-2 has also been shown to regulate enzymes in the glycolytic pathway in

the absence of HIF-1, and HIF-1 is capable of activating some MMPs, suggesting that under some circumstances, HIF-1α and HIF-2α can substitute for each other.

Both of HIF isoforms have common target genes, such as the vascular endothelial growth factor A (*VEGFA*) and glucose transporter 1 (*GLUT1*). Thus, the ability of HIF-1 and HIF-2 to activate specific target genes appears to be dependent on the context [50].

The temporal regulation of HIF-1 and HIF-2 is dependent on the PHDs that regulate HIF-1/2α stability. The actions of the PHDs on different HIF isoforms are generally not equivalent. It has been shown that PHD2 has relatively more influence on HIF-1α than HIF-2α, and PHD3 has relatively more influence on HIF-2α than HIF-1α [62]. Moreover, Koivunen and coworkers showed that FIH may act less effectively on HIF-2α compared to HIF-1α [63]. Hu et al suggested that the differences in the coding sequences of the HIF-α isoforms might also contribute to isoform-specific function [64]. Stiehl et al observed in a MCF7 breast cancer cell model a reciprocal induction kinetic of HIF-α isoforms. HIF-1α expression levels were expressed earlier under hypoxia in comparison to HIF-2α. After a longer time period HIF-2α levels increased, leading to a decreased expression in HIF-1α levels [65] Taking together, we can think that different mechanisms define the activation of HIF-α isoforms in a context dependent manner in response to variations in kinetics and oxygen concentrations.

4.5.3 The prolyl-hydroxylases: PHDs

Some years ago it has been discovered that the ODD domain of HIF-1α was hydroxylated at two proline residues (Pro402 and Pro564) by a prolyl-4-hydroxylase [56] [66]. Three mammalian prolyl-4-hydroxylases have been identified [67]. PHDs are dioxygenases which require both oxygen and 2-oxoglutarate as co-substrates. PHDs transfer one oxygen atom onto the proline residues of the HIF-a subunits; the second oxygen atom reacts with 2-oxoglutarate, producing succinate and carbon dioxide [68].

PHDs contain iron bound by two histidine and one aspartic acid residues. In vitro function of PHDs requires oxygen and 2-oxoglutarate as cosubstrates with iron(II) and

vitamin C serving as cofactors. Although vitamin C deficiency is known to cause the collagen-disassembly disease scurvy, it is unclear whether cellular oxygen sensing is similarly affected. Nytko et al., report that vitamin C-deprived Gulo(-/-) knockout mice show normal HIF-dependent gene expression. They observed that hypoxic HIF induction was also essentially normal under serum- and vitamin C-free cell-culture conditions, and suggested that vitamin C is not required for oxygen sensing in vivo. They showed that glutathione was found to fully substitute for vitamin C requirement of all 3 PHD isoforms in vitro [69].

The first discovery of a PHD was made in the worm *Caenorhabditis elegans* and was called egg-laying abnormal-9 (EGL-9) [67]. In mammals, three different isoforms were discovered: PHD1 (HPH3, EGLN2), PHD2 (HPH2, EGLN1) and PHD3 (HPH1, EGLN3). PHD1-3 differ in size, intracellular localization and tissue distribution [70]. The two modified prolines that constitute the ODD interact separately with pVHL complex[71]. Interestingly, *in vitro* experiments showed that the different PHDs have different preferences for the two target prolines in the HIF-ODD [62].

There is an additional fourth PHD-related protein (PH-4) which has been reported to hydroxylate the two critical prolyl residues of the HIFα subunit. However, unlike the other three PHDs which localize to the cytoplasm and nucleus, PH4 was found to be associated with the endoplasmic reticulum (ER) [72] [73].

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4.5.4 Alternative mechanisms stabilizing HIF-1α under normoxic conditions

Besides the above mentioned mechanisms of hypoxic HIF α subunit stabilization, many growth factors and cytokines are known to stabilize HIF-1α under normoxic conditions. These include insulin, insulin-like growth factors 1 and 2, epidermal growth factor, fibroblast growth factor 2, interleukin 1β, tumor necrosis factor α, angiotensin II, thrombin, transforming growth factor β_1, platelet-derived growth factor, and hepatocyte growth factor [68].

Molecular oxygen nitric oxide (NO) is also a mediator of the inflammatory response. NO can regulate HIF-1α accumulation, activity and HIF-1 dependent target

gene expression [74]. NO regulates HIF-1 by modulating the activity of the oxygen-sensor enzymes PHDs and FIH-1. NO dependent HIF-1alpha accumulation under normoxia occurs because of direct inhibition of PHDs and FIH-1 most likely by competitive binding of NO to the ferrous iron in the catalytically active center of the enzymes [74].

4.5.5 HIF-1 target genes involved in vascular biology

The increase in angiogenesis leads to an enhancement in the vascular density and hence to a decreased oxygen diffusion distance. Moreover, blood flow is controlled by modulation of the vascular tone through production of HIF-1 target genes such as iNOS (inducible nitric oxide synthase), HO-1 (heme oxygenase 1), endothelin 1, adrenomedullin, or activation of the α_{1B}-adrenergic receptor. (reviewed in [68]).

The heart is subjected to hypoxic conditions during ischemia-reperfusion injury, atherosclerosis or during heart failure. One of the first experimental studies which tried to investigate HIF-1α stabilization was performed in a HL-1 cardiac muscle cell line, which showed an increase in mRNA and protein levels of HIF-1α after exposure to 1% O_2 after 4 hours [75]. HIF-1 mRNA and protein upregulation was confirmed in both, neonatal hearts and in cardiomyocytes subjected to chronic hypoxia [76] and furthermore, myocardial HIF-1α mRNA expression was shown to be increased in response to acute ischemia or infraction in human myocardial biopsies from patients on coronary artery bypass (CABG) surgery [77].

4.6 Hypoxia and foam cell formation

Accumulation of cytosolic lipid droplets is caused by increased intracellular cholesterol derived from oxLDL uptake. Hypoxia increases lipid accumulation in macrophages even in the absence of lipoproteins because of increased triglyceride biosynthesis [78]. siRNA studies blocking HIF-1α have shown to block foam cell formation indicating a role for HIF-1α in lipid accumulation [79]. However, the underlying mechanism is poorly understood. A recent study highlighted the

importance of Glut-3 for the *de novo* lipogenesis in hypoxia-induced lipid loading of human macrophages [80]. Hypoxia increases glucose uptake through GLUT3 required for lipid synthesis in macrophages which may contribute to foam cell formation in hypoxic regions of atherosclerotic lesions.

Sterol regulatory element binding protein (SREBP) has been shown to be upregulated in response to low oxygen [81], as well as the SREBP cleavage-activating protein [82], liver receptor X (LXR)[83], the adipocyte differentiation-related protein and peroxisome proliferator-activated receptors (PPARs) [84].

Another angiogenic chemokine, interleukin-8 (IL-8), is upregulated by foam cells located in hypoxic areas of rabbit and human atherosclerotic plaques [85]. Secretion of IL-8 leads to the recruitment of smooth muscle, vascular endothelial and T cells into the atherosclerotic plaques. Rydberg and coworkers showed that macrophages exposed to hypoxia up-regulate the expression of lipooxygenase-2, an enzyme implicated in low-density lipoprotein oxidation. Furthermore, Wüst et al., observed that ALOX15B is the mainly expressed 12/15-lipoxygenase in human macrophages and that its expression is induced by IL-4, LPS, and hypoxia [86].

Another evidence which shows that hypoxia contributes to lipid metabolism in atherosclerotic lesions is that under low concentrations very low-density lipoprotein (VLDL) receptors are upregulated on macrophages [87]. The VLDL receptor mediates the uptake of β-VLDL, chylomicron remnant, and lipoprotein LP(α), contributing to foam cell formation.

Several other genes have been shown to be up-regulated by foam cells in response to hypoxia. One of them is oxygen-regulated protein 150 (ORP150), whose expression is restricted to foam cells within atherosclerotic lesions [88]. The precise biological function of ORP150 is not clear yet, but has been suggested to be linked to macrophage survival in hypoxia [89]. This work proposes that ORP150 may protect foam cells from hypoxia induced stress allowing them to carry out their role of cholesterol scavenging and tissue remodeling in atherogenesis.

The macrophage migration inhibitory factor 1 (*MIF)* has also been shown to be highly expressed by foam cells in atherosclerotic lesions [90]. MIF has been shown to mediate proliferation of cultured endothelial cells [91], so it is likely that in plaques it can also promote neovascularization. Moreover, treatment of ApoE-deficient mice with neutralizing anti-MIF antibodies, reduced a variety of inflammatory mediators which are normally associated with atherogenesis, implying that MIF may also play an important role in intima inflammation [92].

Aggregated LDL (agLDL) stimulates foam cell formation by inducing cholesteryl ester accumulation in lipid droplets. It has been demonstrated recently that agLDL can be internalized by the LDL receptor-related protein (LRP1), which has been also recently shown to be hypoxically induced [93].

Many cytokines and enzymes are induced in hypoxic foam cells within human atherosclerotic plaques, such as VEGF [94] , platelet-derived growth factor (PDGF) [95], TNF-α [96], and others. However, the expression of these factors by hypoxic foam cells in comparison to non hypoxic foam cells in vivo has yet to be determined.

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5. Aims of the study

I. Determine the effect of hypoxia on genes involved in lipid metabolism, particularly genes related to cholesterol uptake and efflux.

II. Explore if hypoxia plays a role in lipid uptake and in foam cell formation through the scavenger receptors SRA, Lox-1 and CD36.

III. Investigate cells and pathways linking HIF-1α to lipid metabolism and to scavenger receptor expression under hypoxia.

IV. Characterize human atherosclerotic plaques, for hypoxia markers and scavenger receptors within the tissue oxygen gradients.

Hypoxia differentially regulates scavenger receptors and lipid uptake in macrophages

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Abstract

Aims Advanced atherosclerotic plaques turn hypoxic in their core as the arterial wall thickens and oxygen diffusion capacity becomes impaired. Macrophage-derived foam cells in atherosclerotic lesions play a pivotal role in plaque formation by expressing scavenger receptors that regulate lipid uptake. However, the role of hypoxia in scavenger receptor regulation remains incompletely understood.

Methods and Results Using RT-qPCR, flow cytometry, and immunoblotting, we found that mRNA and protein expression levels of the scavenger receptor A (SRA) and the cluster of differentiation 36 (CD36) were upregulated by oxidized low-density lipoprotein (oxLDL), but decreased following exposure of macrophages to hypoxia. In contrast, lectin-like oxLDL receptor (Lox-1) mRNA and protein levels were upregulated under hypoxic conditions. Flow cytometry confirmed the increased lipid content in macrophages after exposure to 0.2% oxygen and the hypoxia mimetic dimethyloxalylglycine (DMOG). Antibody-mediated blocking of Lox-1 receptor decreased the hypoxic induction of lipid content and oxLDL uptake. RNAi-mediated knock-down of hypoxia inducible factor (HIF)-1α in macrophages altered the expression of SRA and Lox-1 receptors in hypoxia, suggesting that HIF-1α mediates their modulation by hypoxia.

Conclusions Thus, hypoxia differentially regulates the expression of the three oxLDL receptors Lox1, SRA, and CD36. Lox-1 plays a role in hypoxia-induced foam cell formation.

Keywords Atherosclerosis, foam cell, hypoxia-inducible factor, oxLDL

Introduction

Atherosclerosis is the major cause of myocardial infarction and stroke and the leading cause of mortality in Western countries. It results from the interaction between oxidized low-density lipoproteins (oxLDL), activated endothelial cells, monocyte-derived macrophages, and the vessel wall. Transmigration of monocyte-derived macrophages into the subendothelial space is a key step in atherogenesis. Pro-inflammatory macrophages ingest oxLDL via scavenger receptors and become foam cells, thereby promoting plaque formation [1]. Upon plaque thickening with progression of atherosclerosis, the supply of nutrients and oxygen to the inner parts of atherosclerotic plaques becomes diffusion-limited. As the lesions grow in thickness and the number of foam cells in the intima increases, the plaque core becomes gradually hypoxic and macrophage cellular oxygen consumption is inhibited. Sluimer and coworkers demonstrated the presence of hypoxia by pimonidazole staining of advanced atherosclerotic lesions in symptomatic patients [2]. Oxygen measurements *in vitro* and *in situ* indicated that the oxygen partial pressure in the core inversely correlates with the size of the plaque [3].

Hypoxia-inducible factor (HIF) is the main stimulator of oxygen-regulated gene expression. Hypoxia inhibits oxygen-dependent HIF prolyl-4-hydroxylases (PHDs), leading to the rapid stabilization of HIF α-subunits [4]. In macrophages, oxygen sensing and inflammation cross-talk via NF-κB-induced transcription of the *HIF1A* gene [5]. While these two conditions are well known to contribute to tumor progression, much less is known about their role in atherosclerosis. As in solid tumors, hypoxia and inflammation are increased in the center of atherosclerotic plaques, where they trigger angiogenesis and plaque growth. Plaque-derived cytokines, growth factors, and increased endothelial adhesion molecules recruit monocytes to the activated endothelium, promote transmigration to the subendothelial space, and induce their transdifferention into

macrophages. This step is crucial for the progression of atherosclerosis and is associated with up-regulation of pattern-recognition receptors for innate immunity, including scavenger receptors and toll-like receptors. Scavenger receptors internalize a broad range of molecules and particle-bearing molecules with pathogen-like molecular patterns. Bacterial endotoxins, apoptotic fragments and oxLDL particles are all taken up and destroyed via this pathway. If cholesterol-derived uptake of oxLDL particles cannot be sufficiently mobilized from the cell, it accumulates as cytosolic droplets [1]. Ultimately, macrophages are transformed into foam cells, a hallmark of atherosclerotic lesions.

It has been previously reported that exposure of human macrophages to hypoxia activates proinflammatory pathways such as the Akt and the beta-catenin pathway[6]; moreover, hypoxia favors accumulation of lipid droplets and HIF-1α knock-down inhibits foam cell formation *in vitro* [7; 8]. Foam cell formation is a characteristic event for both early and late atherosclerotic lesions [9]. Although a number of proteins may contribute to this process, scavenger receptors A (SRA), lectin-like oxLDL receptor (Lox-1) and cluster of differentiation 36 (CD36) have been demonstrated to be most relevant [10]. In retinal pigment epithelial cells, mRNA and protein levels of CD36 have recently been shown to be upregulated by hypoxia [11]. Furthermore, decreased SRA upregulation by oxLDL and increased LDL receptor-related protein-1 (LRP1), respectively, have been found after hypoxic exposure [12].

Taken together, the data regarding foam cell formation and the associated pathways in hypoxic macrophages remain conflicting. Thus, we explored the mechanisms by which hypoxia affects the expression of the three main scavenger scavenger receptors SRA, Lox-1, and CD36. We provide evidence for a differential expression of these three scavenger receptors in hypoxic macrophages, thereby demonstrating the importance of impaired oxygen supply for the progression of atherosclerotic plaques.

Methods

Cell culture

Mouse RAW264.7 leukemic monocyte cells and Hepa1 hepatoma cells were grown in DMEM (Sigma). Media were supplemented with 10% fetal calf serum (FCS) and antibiotics (50 IU/ml penicillin and 100 μg/ml streptomycin; Sigma-BRL). If not indicated otherwise, hypoxic cell culture was carried out at 0.2% O_2 using a gas-controlled Invivo$_2$ 400 workstation (Ruskinn Technologies). Cells were treated with 1 mM of the pan PHD inhibitor dimethyloxalylglycine (DMOG) or dimethylsulfoxide (DMSO) as solvent control. RAW264.7 cells were treated with 10 μg/mL oxLDL, acLDL, DiL-oxLDL or DiL-acLDL (KalenBiomed) as indicated.

Protein extraction and immunoblot analysis

Total soluble cellular proteins were extracted with a high salt extraction buffer containing 0.4 M NaCl, 0.1% Nonidet P-40, 10 mM Tris-HCl pH 8.0, 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride and protease inhibitor cocktail (Sigma). Protein concentration was determined by the BCA method and 50 to 70 μg of cellular protein was subjected to immunoblot analysis. PVDF membranes were probed using the following dilutions of mouse monoclonal (mAb), rat monoclonal (ratAb) or rabbit polyclonal (rAb) antibodies: mAb HIF-1α (1:1000; BD Transduction Laboratories), ratAb SRA (1:1000 AbD Serotec), mAb β-actin (1:5000; Sigma), rAb PHD2 (1:1000; Novus), rAb Lox-1 (1:1000; R&D Systems), rAb Glut-1 (1:1000; Abcam) or rAb CD36 (1:1000; Abcam), followed by secondary HRP-conjugated antibodies (1:2000; Pierce). Chemiluminescence signals were developed using Supersignal West Dura substrate (Pierce) and images were acquired with a digital light imaging system (LAS 4000; Fuji).

mRNA quantification

Total RNA purification and mRNA determination by reverse-transcription quantitative PCR (RT-qPCR) using SYBR Green qPCR reagent kit (Sigma) in combination with an MX3000P light cycler (Stratagene) has been described previously [13]. Initial copy number of each sample was calculated by comparison with serial dilutions of a calibrated standard. β-Actin mRNA was used as a housekeeping control for mouse cell lines. Primer sequences are listed in supplemental Table 1.

Plasmids and siRNA transfection

Cells were transfected with 100 nM siRNA duplex oligonucleotides (Invitrogen) by electroporation (Amaxa Biosystems). Therefore, pelleted cells were resuspended in fresh medium and counted using a ViCell counter (Coulter). Per transfection, 4 x 10^6 cells were transferred to a fresh tube and centrifuged at 90 x·g for 10 minutes. After carefully removing the supernatant, the cell pellet was resuspended in 100 μL Mouse Macrophage Nucleofector Solution with supplement (Amaxa Biosystems). siRNA (2 μg) or endotoxin-free pmaxGFP plasmid DNA (Amaxa Biosystems) as transfection control was added and the cells electroporated (Amaxa Nucleofector II, program D-032). Immediately afterwards, cells were diluted in 0.2 mL of DMEM and transferred to 10 cm cell culture dishes. The following forward siRNA oligonucleotides were used: HIF-1α siRNA, 5′-UCUCCAAGCAUCUUCUCAAUGUUUC-3′; control siRNA, 5′-UAAUACUCUGGUAGUUCUCCGGAGC-3′.

Short hairpin RNA constructs and lentiviral infections

Expression vectors encoding short hairpin RNA (shRNA) sequences targeting mouse HIF-1α and a noncoding control driven by the U6 promoter in a pLKO.1-puro plasmid were purchased from Sigma. Viral particles were produced in HEK293T cells using the ViraPower lentiviral expression system according to the manufacturer's instructions (Invitrogen).

Flow cytometry

Single cell suspensions were incubated with antibodies against SRA, Lox-1 and CD36. Immune complexes were visualized by FACScanning using goat anti-rabbit antibody-Alexa 488 for CD36 and Lox-1, or with goat anti-mouse antibody-Alexa 488 for SRA (Molecular Probes, Invitrogen). For lipid uptake experiments, RAW264.7 cells were seeded in six-well dishes, starved with 0.2% FCS in DMEM for 24 hours, and incubated with or without 10 μg/mL oxLDL, DiL-oxLDL, DiL-acLDL or acLDL for 24 hours at 21% or 0.2% oxygen. To block Lox-1, RAW264.7 cells were pre-incubated with 5 μg/mL anti-Lox-1 blocking antibody (RD systems) for 24 hours. Lipids were stained with 1-2 μM 1,1'-dioctadecyl-6,6'-di(4-sulfophenyl)-3,3,3',3'-tetramethylindocarbocyanine (SP-DiIC18(3)) fluorescent probe (DiL) or 3,3´ -dioctadecyloxacarbocyanine perchlorate (DiO) for 4 hours. The cells were washed twice with PBS and analysed with a FACSCantoII and FACSDiva software (BD Pharmingen). Post-acquisition analysis was done with FlowJo7 software (Tree Star). Alternatively, DiL-stained lipids were visualized by fluorescent microscopy using an Olympus BX51 microscope and analySIS software (Olympus).

Lipoprotein uptake

RAW264.7 cells were starved for 24 hours and then incubated with 10 μg/mL oxLDL for 24 hours under normoxic or hypoxic conditions as described above. After washing, cells were fixed with 4% paraformaldehyde and stained with Oil red O (ORO; Sigma). ORO staining was analysed by light microscopy and quantified using analySIS software (Olympus). LDL uptake was quantified as the ratio between the ORO-positive area divided by the number of cells.

Statistics

If not otherwise indicated, results are presented as mean values ± standard error of the mean of at least three independent experiments. Column statistics applying paired Student's *t*-tests were calculated using GraphPad Prism version 4.0 (GraphPad Software).

Results

Hypoxia regulates genes involved in cholesterol transport

To elucidate whether hypoxia affects the expression of genes involved in cellular cholesterol flux, wild-type Hepa1 or HIF-β mutant Hepa1C4 mouse hepatoma cells as well as RAW264.7 mouse macrophages were analyzed for oxygen-dependent expression of candidate genes involved in cholesterol transport. Exposure of these cell lines to pro-inflammatory oxLDL under normoxic (20% O_2) or hypoxic (0.2% O_2) conditions for 24 hours revealed that hypoxia increased mRNA levels of candidate genes such as Lox-1, ABCA1 and ABCG1 cholesterol transporters, sterol-regulated element binding protein (SREBP) and SREBP cleavage activating protein (SCAP), but decreased mRNA levels of SRB, SRA and CD36 (*Figure 1A*). Experiments performed in Hepa1C4 cells suggested that the increased expression of SREBP, SCAP, ABCA1, ABCG1 is HIF-dependent, whereas Lox-1 induction is HIF-independent (*Figure 1A*). Hypoxia attenuated oxLDL-induced SRA, CD36, and SRB, but only SRB attenuation appeared to be at least partially HIF-dependent (*Figure 1A*). The established HIF target gene PHD3 was included as positive control.

Using immunoblotting (*Figure 1B*) and FACS analysis (*Figure 1C*) we could confirm these findings on the protein level. SRA was not detectable in Hepa1 cells (data not shown) and SRA induction by oxLDL in RAW264.7 cells was less clear on the total protein than on the mRNA level (*Figure 1B*). However, surface SRA was again induced by oxLDL under normoxic but not hypoxic conditions (*Figure 1C, left panel*). Hypoxia also attenuated total CD36 protein (*Figure 1B*) but, surprisingly, induced surface CD36 levels (*Figure 1C, right panel*).

HIF-1α mediates hypoxic induction of Lox-1

In contrast to SRA and CD36, we found a robust hypoxic induction of Lox-1 mRNA in Hepa1 and RAW264.7 cells. On the protein level, both hypoxia and DMOG, but not oxLDL or acLDL, induced Lox-1 in RAW264.7 cells (*Figure 2A*). FACS experiments confirmed the hypoxic induction of Lox-1 surface protein expression (*Figure 2B*).

The involvement of HIF-1α in the hypoxic regulation of scavenger receptors was assessed by RNA interference in RAW264.7 cells. Lentiviral transduction with shRNA expression vectors yielded a robust HIF-1α downregulation with shHIF-1α but not with a control shRNA (*Figure 2C*). HIF-1α knock-down abolished hypoxic Lox-1 induction and prevented SRA, but not CD36 reduction (*Figure 2C*).

Hypoxia and DMOG increase lipid content in RAW264.7 macrophages

The differential regulation of the three main scavenger receptors does not allow any prediction of how lipid uptake and content change under hypoxic conditions. Therefore, lipid content was determined in RAW264.7 cells by the fluorescent dye DiL following exposure to 0.2% oxygen, DMOG and/or oxLDL for 24 hours. DiL and DiO stained cells were analysed by fluorescent microscopy (*Figure 3, upper left panels*) and flow cytometry (*Figure 3, middle panels*). Oil Red O (ORO) staining was visualized by light microscopy and used for comparison (*Figure 3, lower left panels*). Both hypoxia (*Figure 3A*) and DMOG (*Figure 3B*) increased the lipid content in RAW264.7 cells already in the absence of oxLDL. Exposure to oxLDL further increased the lipid content (*Figure 3C*).

Lox-1 is necessary for lipid uptake in RAW264.7 cells

To assess the role of hypoxic Lox-1 induction, RAW264.7 cells were treated with anti-Lox-1 blocking antibodies prior to exposure to hypoxia. Total lipid content was determined by DiL (*Figure 4A*) and ORO staining (*Figure 4B*), as well as by DiO staining and flow cytometry (*Figure 4C, left panel*). To analyse lipid uptake directly, RAW264.7 cells were treated with fluorescently labeled DiL-oxLDL, exposed to 0.2% oxygen, and evaluated by flow cytometry (*Figure 4D, left panel*). Demonstrating the relevance of Lox-1, both hypoxia induced lipid content and uptake was attenuated by anti-Lox-1 blocking antibodies.

Discussion

Foam cell formation, a hallmark of atherogenesis, is likely to occur under hypoxic conditions as a consequence of decreased oxygen diffusion to the core of the plaque. Lipids in macrophages include cholesteryl esters and triglycerides which are stored in lipid droplets within the cytosol. Lipid accumulation is mainly due to the uptake of modified lipoproteins, such as oxLDL and acLDL. Hypoxia has been previously reported to lead to lipid accumulation in macrophages even in the absence of lipoproteins [7], and Glut3, a downstream target of HIF-1, has been suggested to be required for *de novo* lipogenesis in hypoxia-induced lipid loading of human macrophages [14]. Furthermore, fatty acid synthase is upregulated by hypoxia via Akt and sterol regulatory element binding protein-1 [15], suggesting a contribution of lipid synthesis to hypoxic foam cell formation.

We found induction of both, lipid content and oxLDL uptake following exposure of RAW264.7 macrophages to hypoxia. Our data suggest a hypoxic switch from SRA and CD36 to the Lox-1 scavenger receptor that increases on the mRNA and protein levels, and which mediates at least in part the increased lipid uptake under hypoxic conditions

While HIF-1α is usually not directly involved in gene repression, hypoxic SRA but not CD36 downregulation were HIF-1α-dependent. SRA protein regulation was further studied by the pan-PHD inhibitor DMOG which also reduced SRA mRNA and protein levels in RAW264.7 cells (data not shown). The hypoxic SRA reduction persisted for 24 hours of milder hypoxia (0.5% or 1% oxygen), and SRA levels partially recovered after 48 hours hypoxia (data not shown). Since HIF-1α levels were also reduced after 48 hours hypoxia, these data additionally support an involvement of HIF-1α in SRA repression. A putative hypoxia response element (HRE) was found in the SRA promoter at position -578 (data not shown), but whether HIF-1 represses SRA by direct promoter binding is currently

unknown. In line with our results, SRA protein levels have been shown to be inhibited by 1% O_2 and $CoCl_2$ in RAW264.7 cells [12].

CD36 protein was slightly reduced under hypoxic conditions, whereas flow cytometry experiments demonstrated an induction on the cell surface, suggesting CD36 relocalization in hypoxia. Evidence for hypoxic CD36 translocation to the plasma membrane has been found in cardiac myocytes [16]. However, conflicting data have been reported about the hypoxic regulation of total CD36: whereas CD36 was increased under low oxygen concentrations in human dermal microvascular endothelial cells, human retinal pigment epithelial and pulmonary artery smooth muscle cells [11], other studies demonstrated a decrease of CD36 expression following hypoxic exposure of adipocytes [17]. Therefore, hypoxic CD36 regulation is likely to be cell type specific.

To the best of our knowledge, our study is the first one to demonstrate the importance of hypoxia-induced Lox-1 for lipid uptake in macrophages. Hypoxia-reoxygenation of cardiomyocytes has been reported to induce Lox-1 [18], but there are no other previous studies on the effects of hypoxia on Lox-1 expression. Lox-1 was first identified as a scavenger receptor for binding and uptake of oxLDL in endothelial cells, and it has been shown that Lox-1 plays important roles in pro-inflammatory signaling and atherogenesis [19]. A cross-talk between hypoxia and inflammation has been further supported by the finding that both TNF-α and NF-κB upregulate Lox-1 expression [20].

We found Lox-1 to be required for uptake of oxLDL under hypoxic conditions. However, hypoxia only slightly stimulated the uptake of acLDL which was not affected by treatment with a blocking anti-Lox-1 antibody (data not shown). These observations suggest that Lox-1 mediates mainly to the uptake of LDL oxidatively modified by an inflammatory milieu. Bioinformatic analysis identified a putative HRE in the Lox-1 promoter at position -114 (data not shown) which might mediate the HIF effect.

In hypoxic macrophages Lox-1 is upregulated, whereas SRA and CD36 are decreased. Thus, we propose that HIF-1α is involved in both SRA inhibition and in Lox-1 upregulation, thereby enhancing oxLDL uptake.

We speculate that with progression of atherosclerotic lesions, the core of the human plaques becomes gradually more hypoxic, accelerating foam cell formation in thick atheromata during advanced stages of atherogenesis. Pharmacologic inhibition of Lox-1 may be an attractive therapeutic option for inhibition of foam cell formation – with and without hypoxia – i.e. during early and late stages of atherogenesis.

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Conflict of interest

None declared

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Figure legends

Figure 1 Expression patterns of candidate genes involved in oxygen-regulated cholesterol transport. (*A*) Heat maps showing the transcript levels of wild-type Hepa1 and HIF-β mutant Hepa1C4 as well as RAW264.7 cells exposed to pro-inflammatory oxLDL at the indicated oxygen concentrations for 24 hours. Transcript levels were determined by RT-qPCR and normalized to β-actin mRNA levels. (*B*) Immunoblotting and (*C*) FACS analysis of SRA and CD36. RAW264.7 cells were exposed to oxLDL and/or DMOG as indicated and exposed to 21% or 0.2% O_2 for 24 hours. DMSO was used as solvent control.

Figure 2 HIF-1α-mediated upregulation of Lox-1 in hypoxia. (*A*) Immunoblotting of CD36 and Lox-1 in RAW264.7 cells exposed to oxLDL or acLDL and/or 0.2% oxygen or DMOG for 24 hours. (*B*) FACS analysis of Lox-1 expression on RAW264.7 cells exposed to oxLDL and/or 0.2% oxygen. (*C*) Immunoblotting of SRA, CD36, and Lox-1 in RAW264.7 cells following shRNA-mediated knock-down of HIF-1α (shHIF-1α) and treatment as above. Lentiviral particles containing a noncoding shRNA were used as control (shControl). DMSO was used as solvent control in all experiments.

Figure 3 Increased lipid content in macrophages under hypoxic conditions. RAW264.7 cells were starved in 0.2% FCS for 24 hours, followed by exposure to (*A*) hypoxia, (*B*) DMOG or DMSO solvent control, or (*C*) oxLDL with and without hypoxia. Lipid staining was performed with DiL (4 hours) or Oil Red O (ORO) (24 hours) as indicated, and detected by fluorescence and light microscopy, respectively (*left panels*) DiL fluorescence was also quantified by FACS (*right panels*) and evaluated using the folds

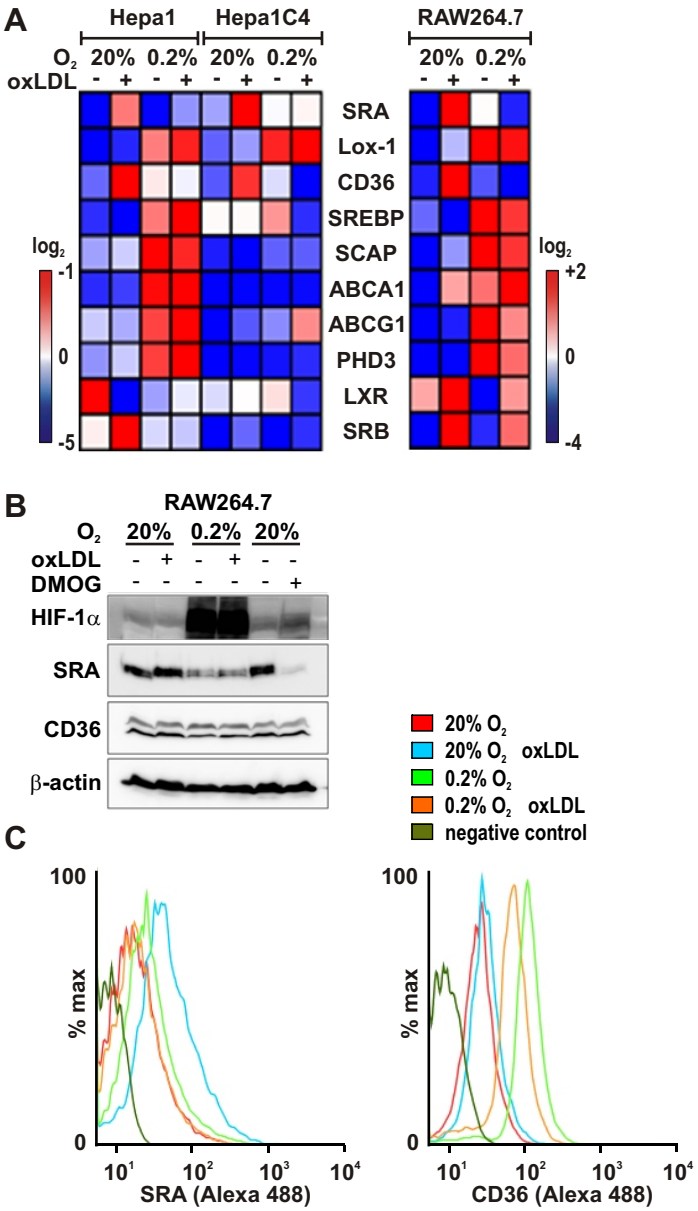
of induction of median fluorescence values. Oil red O and fluorescent microscopy data were quantified dividing positive stained area per number of cells. Results are presented as mean values ± standard error of the mean of at least three independent experiments. Column statistics applying paired Student's *t*-tests were calculated using GraphPad Prism version 4.0 (GraphPad Software). FACS data were quantified and statistically evaluated using the folds of induction of median fluorescence values.

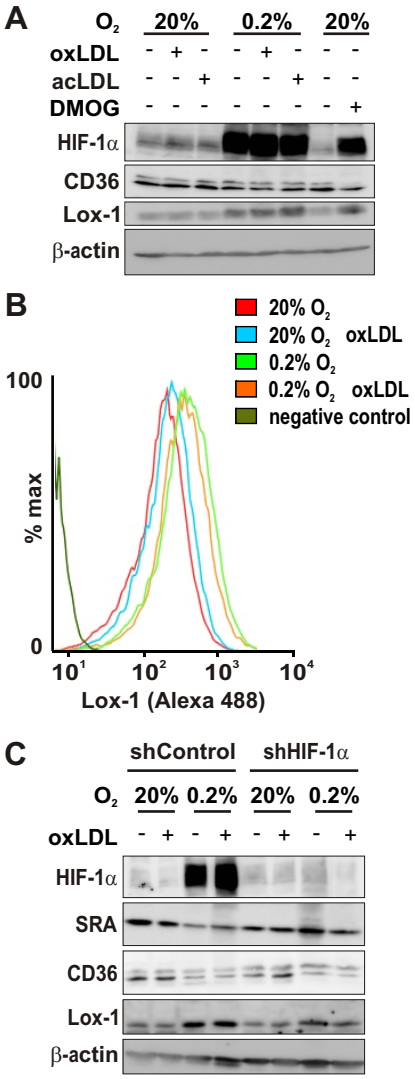
Figure 4 Requirement of Lox-1 for lipid uptake in RAW264.7 macrophages. RAW264.7 cells were starved in 0.2% FCS for 24 hours, and incubated with or without oxLDL for 24 hours at 20% or 0.2% oxygen, respectively. Where indicated, Lox-1 was blocked previously by treatment with anti-Lox-1 antibodies for 24 hours. To determine lipid content, the cells were stained with DiO (*A*) or Oil Red O (ORO) (*B)* for 4 and 24 hours respectively, and analyzed by fluorescence microscopy (*A*), light microscopy (*B*) and FACS (*C*), respectively. (*D*) To determine lipid uptake, untreated or anti-Lox-1 treated RAW264.7 cells were incubated with DiL-labelled oxLDL (DiL-oxLDL) for 24 hours and analyzed by flow cytometry. (*C, D*) Oil red O and fluorescent microscopy data were quantified dividing positive stained area per number of cells. Results are presented as mean values ± standard error of the mean of at least three independent experiments. Column statistics applying paired Student's *t*-tests were calculated using GraphPad Prism version 4.0 (GraphPad Software). FACS data were quantified and statistically evaluated using the folds of induction of median fluorescence values (*right panels*).

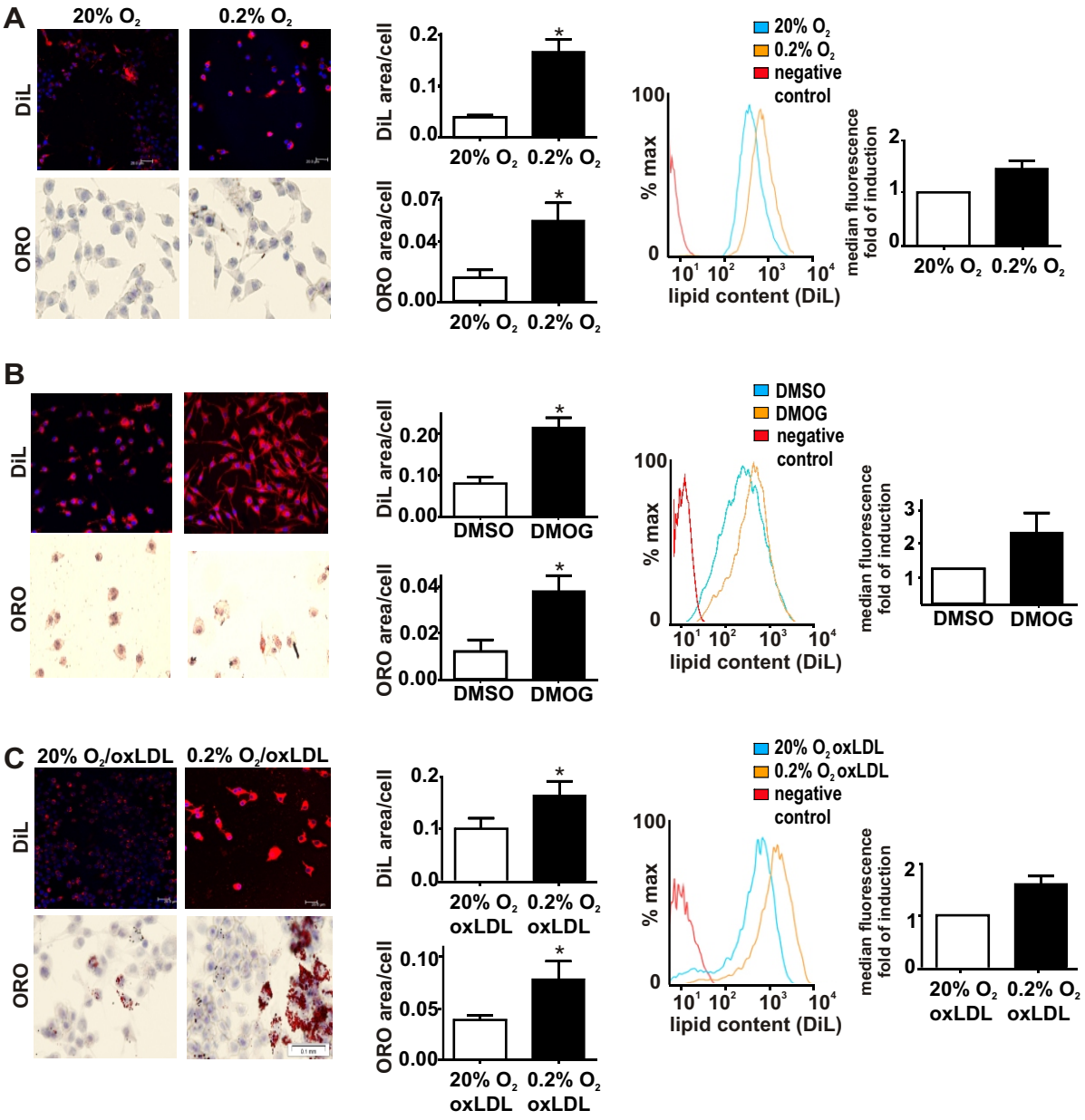
Supplementary data

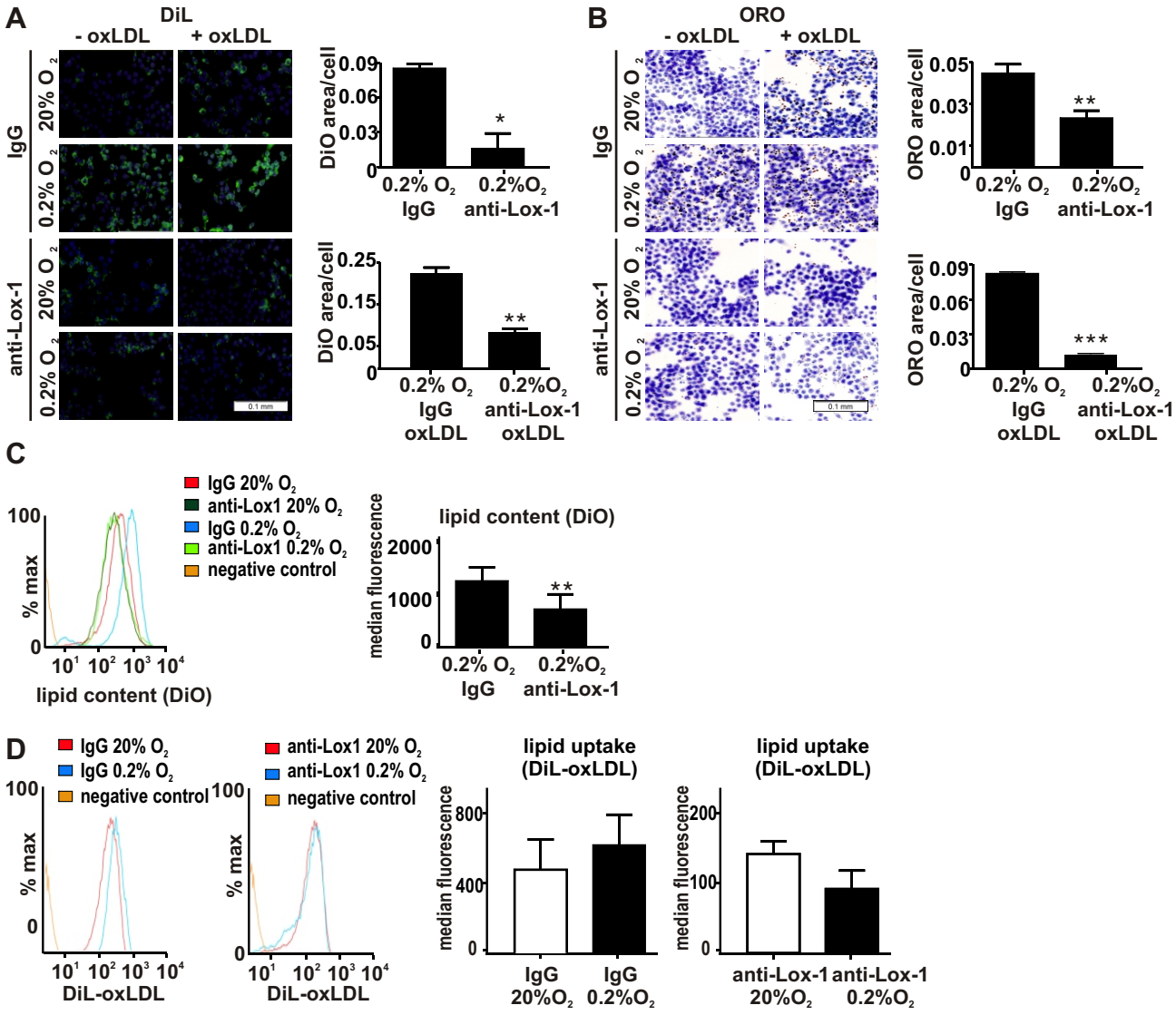
Supplemental Table 1. Primers used for RT-qPCR to quantitate mRNA levels of genes involved in oxygen regulated cholesterol uptake and efflux.

Lox-1	F	TCTTCCATGGGCCCTTTAGCTG
Lox-1	R	TTCCGATGCAATCCAATCCAGA
LXR	F	AGCAGCTGGGCATGATCGAGAA
LXR	R	AAGTGGGCAAAGCGCTGTTGTC
Scap	F	CCTGCAGGTTTGGTGCGCCT
Scap	R	GGGGCTTGCCACCCATTGCT
SR-A	F	AAAGAAGAACAAGCGCACGTGG
SR-A	R	GAGCACCAGGTGGACCAGTTTG
CD36	F	TCCTATTGGCCAAGCTATTGCG
CD36	R	CACGGGGATTCCTTTAAGGTCG
ABCA1	F	ATAGTGTGGAGCTGCCCCATCA
ABCA1	R	CCACATCCTGCAAGTAGGCGAA
PHD3	F	CAACTTCCTCCTGTCCCTCA
PHD3	R	GGCTGGACTTCATGTGGATT
ABCG1	F	GATTGG GAATGAAGCCAAGA
ABCG1	R	GCTGGGACGTCATCCAGTAT
SREBP	F	CACGGTACCAGCAATGGAC
SREBP	R	CAGATAGCAGGATGCCAACA
β-Actin	F	GAGCGTGGCTACAGCTTCAC
β-Actin	R	GGCATAGAGGTCTTTACGGATG


A
Hepa1
Hepa1C4
RAW264.7
O2 20% 0.2% 20% 0.2%
oxLDL - + - + - + - +
SRA
Lox-1
CD36
SREBP
SCAP
ABCA1
ABCG1
PHD3
LXR
SRB
log2
-1
0
-5
+2
-4
B
RAW264.7
O2 20% 0.2% 20%
oxLDL - + - + - -
DMOG - - - - - +
HIF-1α
SRA
CD36
β-actin
20% O2
20% O2 oxLDL
0.2% O2
0.2% O2 oxLDL
negative control
C
100
% max
0
10^1 10^2 10^3 10^4
SRA (Alexa 488)
CD36 (Alexa 488)


A
O2
20%
0.2%
20%
oxLDL
acLDL
DMOG
HIF-1α
CD36
Lox-1
β-actin
B
20% O2
20% O2 oxLDL
0.2% O2
0.2% O2 oxLDL
negative control
100
0
% max
10^1
10^2
10^3
10^4
Lox-1 (Alexa 488)
C
shControl
shHIF-1α
O2
20%
0.2%
20%
0.2%
oxLDL
HIF-1α
SRA
CD36
Lox-1
β-actin


A
20% O2
0.2% O2
DiL
ORO
DiL area/cell
0.2
0.1
0.0
20% O2 0.2% O2
ORO area/cell
0.07
0.04
0.00
20% O2 0.2% O2
20% O2
0.2% O2
negative control
100
% max
0
10^1 10^2 10^3 10^4
lipid content (DiL)
median fluorescence fold of induction
2
1
0
20% O2 0.2% O2
B
DMSO
DMOG
DiL area/cell
0.20
0.10
0.00
DMSO DMOG
ORO area/cell
0.04
0.02
0.00
DMSO DMOG
DMSO
DMOG
negative control
100
% max
0
10^1 10^2 10^3 10^4
lipid content (DiL)
median fluorescence fold of induction
3
2
1
DMSO DMOG
C
20% O2/oxLDL
0.2% O2/oxLDL
DiL
ORO
DiL area/cell
0.2
0.1
0.0
20% O2 oxLDL
0.2% O2 oxLDL
ORO area/cell
0.10
0.05
0.00
20% O2 oxLDL
0.2% O2 oxLDL
20% O2 oxLDL
0.2% O2 oxLDL
negative control
100
% max
0
10^1 10^2 10^3 10^4
lipid content (DiL)
median fluorescence fold of induction
2
1
0
20% O2 oxLDL
0.2% O2 oxLDL


A
DiL
- oxLDL
+ oxLDL
IgG
anti-Lox-1
20% O2
0.2% O2
DiO area/cell
0.2% O2 IgG
0.2%O2 anti-Lox-1
0.2% O2 IgG oxLDL
0.2%O2 anti-Lox-1 oxLDL
B
ORO
ORO area/cell
C
IgG 20% O2
anti-Lox1 20% O2
IgG 0.2% O2
anti-Lox1 0.2% O2
negative control
% max
lipid content (DiO)
median fluorescence
D
DiL-oxLDL
lipid uptake (DiL-oxLDL)
IgG 20%O2
IgG 0.2%O2
anti-Lox-1 20%O2
anti-Lox-1 0.2%O2

6.2 Manuscript II

Expression of 12/15-lipoxygenases in human primary macrophages

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Expression and regulation of 12/15-lipoxygenases in human primary macrophages

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ABSTRACT

Objectives: Atherosclerosis is a chronic disease characterized by two main features, lipid retention and inflammation. The 12/15-lipoxygenases play a two-faced role in atherosclerosis with pro-inflammatory effects through oxidation of LDL and anti-inflammatory effects through lipid mediator synthesis. In cells involved in atherosclerosis the 12-lipoxygenase ALOX12 and the two 15-lipoxygenases, ALOX15 and ALOX15B may be expressed but their expression has not yet been investigated in detail.
Methods: To investigate the regulation of ALOX12, ALOX15 and ALOX15B in human macrophages we measured basal mRNA and protein expression during differentiation of monocytes to macrophages and stimulated expression in macrophages.
Results: The results show an increase of ALOX15B during the differentiation of monocytes to macrophages, while the expression of ALOX12 and ALOX15 remains on the same low level. Stimulation of macrophages with a set of cytokines and with hypoxia revealed that IL-4, IL-13, LPS and hypoxia further increase the ALOX15B mRNA. Western blot analysis showed that IL-4, LPS and hypoxia increase the ALOX15B protein expression, whereas IL-13 has no effect on the protein levels. IL-4 and IL-13 also enhance ALOX15 mRNA and protein expression, whereas none of the stimuli has an impact on ALOX12 expression.
Conclusion: In summary, these data suggest that ALOX15B is the mainly expressed 12/15-lipoxygenase in human macrophages and that its expression is induced by IL-4, LPS and hypoxia. IL-4 and IL-13 also increase the expression of ALOX15, however, only IL-4 stimulation seems to drive ALOX15 expression to levels higher than the basal expression of ALOX15B. Hence, ALOX15B may play a major role in human atherosclerosis.

1. Introduction

There is compelling evidence for an anti-inflammatory effect of 12- and 15-lipoxygenases through the generation of lipid mediators involved in the resolution of inflammation [1]. On the other hand, there is compelling evidence for a pro-atherosclerotic effect through the formation of oxLDL which accelerates foam cell formation and through its role in signalling of angiotensin II mediated mechanisms and vascular smooth muscle cell proliferation [1]. Hence, the 12/15-lipoxygenases seem to be two-faced enzymes with an anti-inflammatory effect through lipid mediator production, and a pro-inflammatory and atherogenic effect through oxLDL formation and participation in signalling pathways [1].

Animal models of atherosclerosis did not solve the question of whether the 12/15-lipoxygenase activity is pro- or anti-atherogenic because different animal models showed contrasting results [2–7]. Monocyte specific 15-lipoxygenase expression in transgenic rabbits reduced atherosclerosis and supported the anti-inflammatory role of the 15-lipoxygenase [2,3]. Similarly, an extensive mouse study applying several overexpressing and knockout mouse lines showed an atheroprotective effect of the 15-lipoxygenase under a normal diet [7]. However, conditional macrophage-specific and general disruption of the mouse homolog 12-lipoxygenase gene reduced atherosclerosis [4,5], while overexpression of human 15-lipoxygenase in vascular endothelium enhanced atherosclerosis in other mouse strains on a cholesterol rich diet [6]. The discrepancies between the different animal models have been explained by the

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different positional selectivities of the mammalian 12- and 15-lipoxygenase iso-enzymes which oxidize arachidonic acid at the carbon atoms 12 and 15 and which have different expression patterns, and by the composition of the food used in these animal studies [1,8].

To investigate the role of the 12/15-lipoxygenases in human atherosclerosis, genetic studies have been carried out which investigated the association of the human *ALOX15* gene with coronary artery disease and myocardial infarction [9–11]. Although there is currently more support for a neutral or an atheroprotective role of *ALOX15* than for the contrary, these human genetic studies did not consistently show an association of functional variants in *ALOX15* with clinical end points of atherosclerosis [12]. The lack of consistent associations may be explained by the lack of power of the studies due to the low frequency of the two functional polymorphisms [12]. However, another explanation may be redundancy for the 12/15-lipoxygenase activity in human macrophages. Recently a second 15-lipoxygenase isoform, ALOX15B, was detected in human atherosclerotic plaques [13,14]. Immunohistochemical analyses showed abundant ALOX15B expression in macrophage-rich areas of carotid lesions, and lipidomic analyses demonstrated the presence of typical ALOX15B products in plaque tissue [15].

These findings suggest that eventually more than one 12/15-lipoxygenase isoform may play a role in human atherosclerosis. Humans have two 12-lipoxygenases and two 15-lipoxygenases which show different expression patterns, substrate specificities and stereo-selective metabolism [1]. However, in cells involved in atherosclerosis only the 12-lipoxygenase, ALOX12, and the two 15-lipoxygenases, ALOX15 and ALOX15B, seem to be expressed [15]. To identify the major 12/15-lipoxygenases in human macrophages and to better understand their role in human atherosclerosis, we investigated the basal and stimulated expression of these three 12/15-lipoxygenase isoforms ALOX12, ALOX15 and ALOX15B in human primary macrophages.

2. Materials and methods

2.1. Material

IL-1β, INF-γ, LPS, Poly I:C, TNF-α and TGF-β were purchased from Sigma–Aldrich (Buchs, Switzerland). IL-4, IL-13, IL-6 and the human M-CSF were obtained from R&D Systems Europe Ltd. (Abingdon, United Kingdom). The TLR9 ligand, CpG-oligodeoxynucleotides (CpG) was synthesized by Microsynth (Balgach, Switzerland). The TLR7 and TLR8 ligands 3M-001 rsp. 3M-002 were purchased from 3M Pharmaceuticals (St. Paul, Minnesota, USA). ALOX15 antibody (Anti-15 Lipoxygenase 1, monoclonal antibody) was ordered from Abcam (Cambridge, United Kingdom), ALOX15B antibody (Anti-15-LOX form 2, polyclonal antibody) from Oxford Biomedical Research (Oxford, United Kingdom) and the β-actin antibody from Sigma–Aldrich.

2.2. Preparation of human peripheral monocytes and cell culture

White blood cells from healthy volunteers were isolated from buffy coat (Blutspendezentrum Zürich, Schlieren, Switzerland) using Histopaque-1077 gradient (Sigma–Aldrich). Peripheral human monocytes were purified by capturing with anti-CD14 antibody coupled to MACS® microbeads (Miltenyi Biotech, Bergisch Gladbach, Germany). Monocytes were seeded with a density of $0.7*10^6$ cells/ml and were cultured for differentiation into macrophages for 7 days at 37 °C and 5% CO_2 in RPMI-1640 (Sigma–Aldrich) supplemented with 5% Fetal Calf Serum (Bioconcepts, Allschwil, Switzerland), 5% Human AB Serum (Sigma–Aldrich), 1% Penicillin/Streptomycin (Invitrogen, Zug, Switzerland). For the stimulation experiments with the set of cytokines, the cells were starved for 24 h in RPMI-1640 (Sigma–Aldrich) containing 1% Penicillin/Streptomycin (Invitrogen) before they were cultured in RPMI-1640 (Sigma–Aldrich) supplemented with 5% Human AB Serum (Sigma–Aldrich), 1% Penicillin/Streptomycin (Invitrogen) and the indicated cytokines for different time points. Monocytes were differentiated to macrophages in the presence of M-CSF (50 ng/ml) for western blot experiments with IL-4 and IL-13 stimulation. For the protein experiment with LPS stimulation human monocytes were differentiated without M-CSF stimulation. All experiments were performed under normoxic conditions, unless indicated. For hypoxic conditions, cells were incubated in a hypoxic chamber at 0.2% O_2 and 5% CO_2 for the indicated times without M-CSF stimulation.

2.3. Quantitative real-time PCR (qPCR)

Total amount of RNA was extracted using RNeasy Mini Kit (Qiagen AG, Hombrechtikon, Switzerland) and the reverse transcription reaction was performed with 0.5 μg RNA in a 20 μl reaction using random primer (Invitrogen) with the Superscript III Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. The qPCR reaction was done on a LightCycler 480 system (Roche Diagnostics, Rotkreuz, Switzerland) utilizing a hot-start SYBR green method with the following parameters: preheating for 10 min at 95 °C, followed by 40 cycles of denaturation for 10 s at 95 °C, annealing for 10 s at 60 °C and extension for 10 s at 72 °C. The quantification PCR included 50 ng cDNA, 0.5 μM primers forward and reverse and 5× SYBR green master mix (Roche Diagnostics). The primers were designed with the OLIGO 6.0 software (Molecular Biology Insights, Inc., Cascade, USA) and the sequences are listed in Table 1. To quantify the 2 variants of ALOX15B, the primer pair ALOX15B-exon 9 was created with the upper primer located in exon 9 to detect the canonical isoform ALOX15B only. To quantify ALOX15Bsv-a, the canonical ALOX15B mRNA was subtracted from the total ALOX15B mRNA obtained with the primer pair ALOX15B, which detects both variants, ALOX15B and ALOX15Bsv-a. Calculation of the absolute copy number was done using individual standard curves and the following equation: copy

Table 1
Sequences of qPCR primers.

Gene	Forward primer	Reverse primer
ALOX12 NM_000697.2	5′AGTTCCTCAATGGTGCCAAC3′	5′GCAGCCAGGTATTGCTTCTC3′
ALOX15 NM_001140.3	5′CTTCAAGCTTATAATTCCCCAC3′	5′GATTCCTTCCACATACCGATAG3′
ALOX15B NM_001141.2	5′CTACAGGCTGGCTCTGCTTT3′	5′GGATCAGGACAGGGTTGAGA3′
ALOX15B-exon 9 NM_001141.2	5′GACAAGTGGGACTGGTTGCT3′	5′TTGATGTGCAGGGTGTATCG3′
GAPDH NM_002046.3	5′CCCATGTTCGTCATGGGTGT3′	5′TGGTCATGAGTCCTTCCACGATA3′
L28 NM_001136134.1	5′GCAATTCCTTCCGCTACAAC3′	5′TGTTCTTGCGGATCATGTGT3′
IL-10 NM_000572	5′GATCCAGTTTTACCTGGAGGAG3′	5′CCTGAGGGTCTTCAGGTTCTC3′
TNF-α NM_000594	5′GAGTGACAAGCCTGTAGCCCATGTTGTAGCA3′	5′GGCAATGATGATCCCAAAGTAGACCTGCCCAGACT3′
IL-1β NM_000576	5′TACCTGTCCTGCGTGTTGAA3′	5′TCTTTGGGTAATTTTTGGGATCT3′

number per µl = amount of original PCR product (ng)*6.022*10^{23}/ number of basepairs of the PCR product*650*10^{9}*dilution factor.

2.4. Western blot analysis

Macrophages cultured in cell culture dishes were washed with phosphate buffer saline solution (GIBCO PBS, Invitrogen) and lysed using ProteoJet™ Mammalian Cell Lysis Reagent (Fermentas, St.Leon-Rot, Germany). Protein concentrations were determined using the ND-1000 Spectrometer Nanodrop (NanoDrop Technologies, Inc., Wilmington, USA). Protein samples were denatured using Laemmli buffer (4% SDS, 10% 2-mercaptoethanol, 20% glycerol, 0.004% bromophenol blue, 0.125 M Tris HCl) and electrophoretically separated on a 4–12% Bis–Tris Gel NUPAGE SDS gel (Invitrogen) and transferred to nitrocellulose membranes (Protran, 0.45 µm, Whatman Switzerland GmbH, Bottmingen, Switzerland). The membranes were blocked with 5% bovine serum albumin (BSA, Sigma–Aldrich) in Tris-buffered saline solution containing 0.2% Tween 20 (TBST) (TBS, 0.2 M Trisbase, 1.5 M NaCl, pH 7.6) for 3 h at room temperature (RT). Membranes were then incubated overnight at 4 °C with anti-alox15 antibody (1:2000) or anti-alox15b antibody (1:2000). After washing in TBST, the blots were blocked for 1 h in a 5% BSA-TBST solution and incubated with horseradish peroxidase (HRP)-conjugated secondary antibody for 2 h at RT (1:10'000, anti-rabbit IgG for alox15b and anti-mouse IgG for alox15 detection, GE Healthcare, Glattbrugg, Switzerland). Following the rinse in TBST the blots were developed using the enhanced chemiluminescence (ECL) kit (GE Healthcare) and exposed to UltraCruz™ Autoradiography Film (Santa Cruz Biotechnology, Heidelberg, Germany). The blots were incubated in Restore Western Blot Stripping Buffer (Fisher Scientific AG, Wohlen, Switzerland) for 15 min at RT and reprobed with antibodies for β-actin (1:10,000). Autoradiographic films were scanned and quantitative analysis of detected peptides was performed by densitometry using the quantity one software (Bio-Rad Laboratories, Hemel Hempstead, United Kingdom).

2.5. FACS analysis

FACS analysis was performed using a monoclonal PE-labeled anti-human CD80, a monoclonal FITC-labeled anti-human CD206 and an IgG1K isotype control (BD Biosciences, San Jose, California USA). Briefly, cells were resuspended in PBS containing 2.5% FCS (Bioconcepts) and incubated in the dark for 30 min at 4 °C before analysis was carried out on a FACS Calibur Analyzer (BD Biosciences, San Jose, California USA).

2.6. Characterization of macrophages

To confirm the polarization of the resting macrophages into the M1 (LPS) and M2 (IL-4, IL-13) subtypes following stimulation, we measured the expression of different markers characteristic for the two subpopulations. LPS stimulation increased the mRNA expression of the pro-inflammatory cytokines IL-1β and TNF-α and the cell surface expression of CD80 (Supplementary Fig. 1). IL-4 and IL-13 stimulation increased the mRNA expression of the anti-inflammatory cytokine IL-10, reduced the mRNA of the pro-inflammatory IL-1β and TNF-α, and increased the surface marker CD206 (Supplementary Figs. 2 and 3).

2.7. Statistical analysis

Statistical analysis was done using the software excel (*t*-test) (Microsoft, Wallisellen, Switzerland) or graphprism 4 (ADNOVA) (GraphPad Prism Software, Inc, La Jolla, USA). Differences between individual groups were calculated using two-tailed unpaired students *t*-test, while one way ADNOVA with the Dunnett's post test was used for time course experiments. Results are shown as mean SD and a *p*-value of <0.05 was considered significant.

3. Results

3.1. mRNA expression of the 12/15-lipoxygenases in the time course of monocyte to macrophage differentiation

To investigate the expression of the 3 different 12/15-lipoxygenases, ALOX12, ALOX15 and ALOX15B, in monocytes and macrophages, the absolute copy number of the different mRNAs was measured in a time course experiment from day 0 to day 7 during the differentiation of monocytes to macrophages. As shown in Fig. 1, the expression of the ALOX15B mRNA increased during the time of differentiation while ALOX12 and ALOX15 expression stayed at the same low level. These results indicate that ALOX15B is expressed in non-stimulated human resident macrophages and that it represents the only 12/15-lipoxygenase in these cells (Fig. 1).

3.2. Expression of ALOX12, ALOX15 and ALOX15B mRNA after stimulation of human macrophages with pro- and anti-inflammatory stimuli

To investigate the regulation of the 3 different 12/15-lipoxygenases, human macrophages were stimulated with different pro- and anti-inflammatory cytokines, TLR agonists or incubated under hypoxic conditions for 24 h, and the relative mRNA expression was measured by qPCR. None of the stimuli had an effect on ALOX12 mRNA expression (Fig. 2A). In contrast, the anti-inflammatory cytokines IL-4 and IL-13 increased ALOX15 mRNA expression in human macrophages (Fig. 2B), similar to the previously described increase in human monocytes [16]. Intriguingly, ALOX15B mRNA expression was stimulated by IL-4, IL-13 and by the pro-inflammatory Toll-like Receptor 4 (TLR4) agonist LPS, while the pro-inflammatory cytokine IL-6 reduced ALOX15B mRNA expression. In addition, we corroborated previous results showing that ALOX15B expression increases upon hypoxic treatment of macrophages (Fig. 2C) [13]. No stimulation of any of the 12/15-lipoxygenases was observed with IL-1β, TNF-α, TGF-β, and with the agonists for TLR3 (Poly I:C), TLR7 (3M-001), TLR8 (3M-002), and TLR9 (CpG).

Since four ALOX15B mRNA isoforms (ALOX15Bsv-a/b/c) have been observed in prostate epithelial cells [17], we first investigated the presence of these isoforms in monocytes and macrophages. As

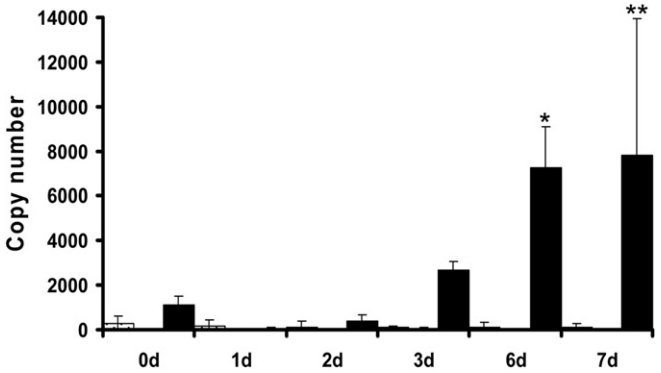

Fig. 1. Absolute copy number of the ALOX12 (dotted), ALOX15 (dashed) and ALOX15B (black) mRNA in the time course of the differentiation of monocytes to macrophages from day 0 to day 7. Bars indicate the mean of 3 independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$.

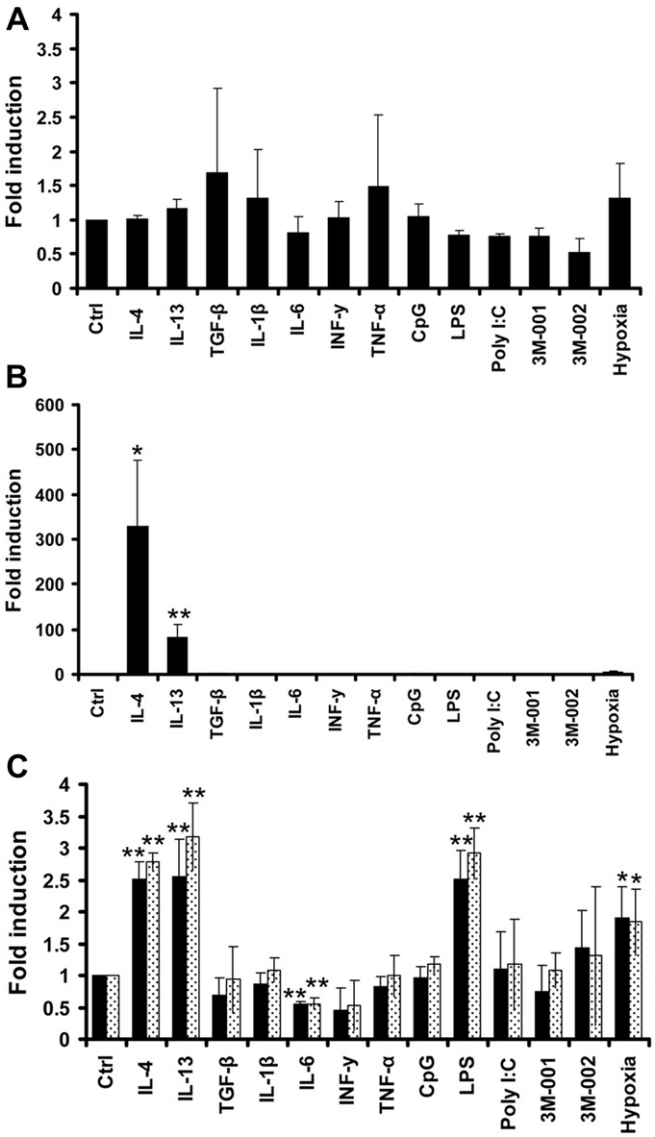

Fig. 2. Relative mRNA expression of ALOX12, ALOX15 and ALOX15B after stimulation of human macrophages with different stimuli for 24 h (IL-4 10 ng/ml, IL-13 10 ng/ml, TGF-β 1 ng/ml, IL-1β 1 ng/ml, IL-6 10 ng/ml, INF-γ 50 ng/ml, TNF-α 1 ng/ml, CpG 100 ng/ml, LPS 100 ng/ml, Poly I:C 1 ng/ml, 3M001 3 μM, 3M002 3 μM). The values were normalized for GAPDH and L28 mRNA expression for the cytokines and TLR agonists, and for hypoxia, respectively. A: ALOX12, B: ALOX15, C: ALOX15B canonical isoform (dotted) and both variants (black), ALOX15B and ALOX15Bsv-a. Bars indicate the mean of 3 independent experiments as fold induction of control. $^{*}p < 0.05$, $^{**}p < 0.01$.

previously observed [13], human macrophages express the canonical isoform and the splice variant ALOX15Bsv-a, which most likely will translate an inactive protein [13]. To investigate whether the relative abundance of these isoforms is altered in macrophages by the stimuli applied, we quantified the two isoforms by qPCR. As shown in Fig. 2C, none of the stimuli altered the splicing pattern of ALOX15B suggesting that differential splicing was not regulated in macrophages under the investigated stimulations.

To estimate which of the two 15-lipoxygenases, ALOX15 and ALOX15B is the major 12/15-lipoxygenase in human M2 macrophages, we compared the absolute copy number of the ALOX15 and the ALOX15B mRNAs following IL-4 and IL-13 stimulation. As shown in Fig. 3 the stimulated expression of ALOX15B is higher in M2 macrophages than ALOX15 expression after stimulation with these two cytokines. Interestingly, only the stimulation of

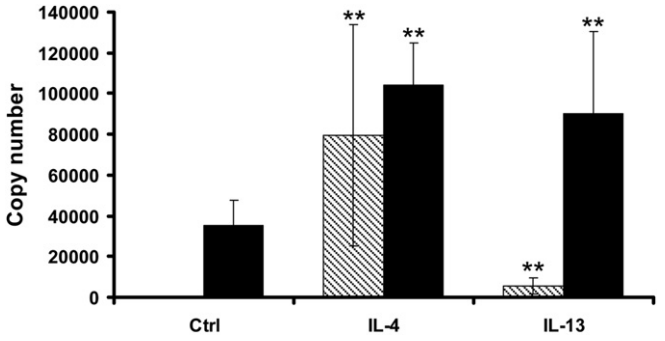

Fig. 3. Absolute mRNA expression of ALOX15 (dashed) and ALOX15B (black) after stimulation of human macrophages with IL-4 and IL-13 for 24 h (IL-4 10 ng/ml, IL-13 10 ng/ml). Bars indicate the mean of 3 independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$.

macrophages with IL-4 increased the ALOX15 mRNA expression to levels higher than ALOX15B expression in untreated cells (Fig. 3). These results indicate that ALOX15B is also a major source of 12/15-lipoxygenase activity in stimulated macrophages and that the mRNA levels are comparable to or even higher than the mRNA levels of ALOX15 in such M2 macrophages.

3.3. ALOX15 and ALOX15B protein expression in a time course experiment with IL-4, IL-13, LPS and hypoxia over 3 days

To test the long term effect of IL-4, IL-13, LPS and hypoxia stimulation on the expression of ALOX15 and ALOX15B, human macrophages were stimulated for 24, 48 and 72 h. IL-4 increased ALOX15 mRNA expression with a significant peak at 48 h (Fig. 4A, black) and IL-13 enhanced the mRNA expression gradually over 3 days with a peak at 72 h (Fig. 4A, dotted). Stimulation of ALOX15 expression by IL-4 could be confirmed on the protein level by western blot analysis (Fig. 4B, black) whereas stimulation with IL-13 only slightly induced protein expression (Fig. 4B, dotted).

In the same time course experiments, ALOX15B mRNA expression gradually increased over 3 days following IL-4, IL-13 and LPS stimulation (Fig. 5A). This rise in ALOX15B was also observed on the protein level following IL-4 and LPS stimulation. Stimulation by IL-4 and LPS supported translation of ALOX15B protein expression in a cumulative way (Fig. 5B, black and dashed) while the stimulation by IL-13 was not translated into protein (Fig. 5B, dotted). To investigate the ALOX15B expression under hypoxia in a time course, human macrophages were incubated for 24, 48 and 72 h under hypoxic conditions. The ALOX15B mRNA showed an increase after 24 h and peaked at 72 h (Fig. 5A, white) while the protein levels peaked after 48 h (Fig. 5B, white).

4. Discussion

In this study we demonstrate that ALOX15B is the only 12/15-lipoxygenase expressed in human resident and hypoxic macrophages and that polarization with LPS further increases ALOX15B expression in M1 macrophages. Only in macrophages stimulated with IL-4, ALOX15B and ALOX15 are expressed, indicating that in such M2 macrophages both isoforms are responsible for the 15-lipoxygenase activity. These results emphasize that in addition to hypoxic resident macrophages and M2 macrophages, a certain set of human M1 macrophages will also express 15-lipoxygenase activity.

In addition to the differentiation of monocytes into resident macrophages, macrophages can be polarized into at least two subpopulations, identified as M1 and M2 macrophages, analogous to the well characterized Th1 and Th2 subpopulations of T-helper

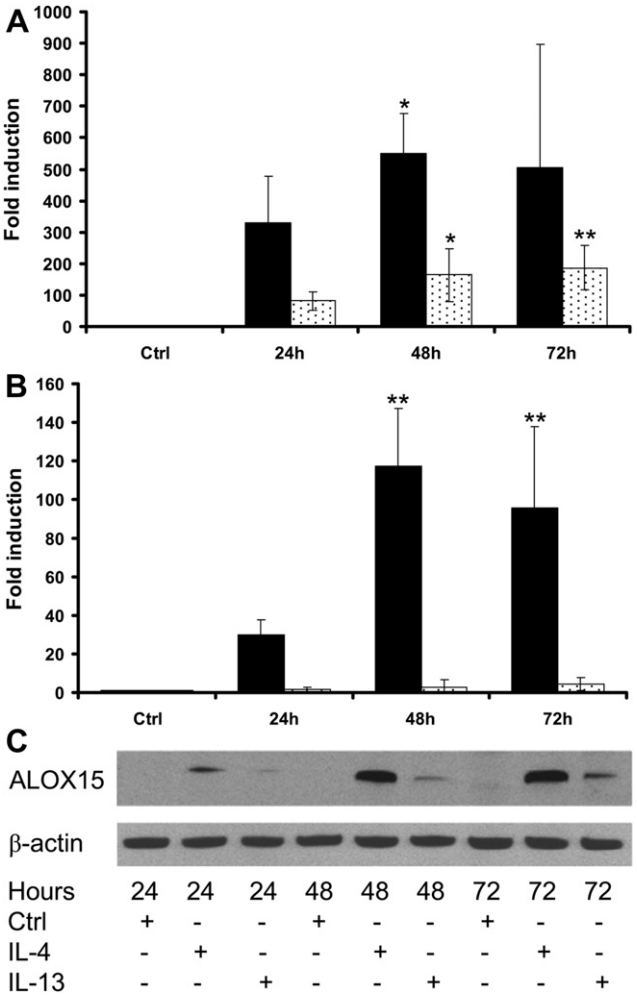

Fig. 4. mRNA and protein expression of ALOX15 after stimulation with IL-4 and IL-13 for 24, 48 and 72 h (IL-4 10 ng/ml, IL-13 10 ng/ml). A: mRNA expression following IL-4 (black) and IL-13 (dotted) stimulation; B: protein expression following IL-4 (black) and IL-13 (dotted) stimulation. C: representative western blot used for the densitometric analysis following IL-4 and IL-13 stimulation. Bars indicate the mean of 3 independent experiments as fold induction of control. $^{*}p < 0.05$, $^{**}p < 0.01$.

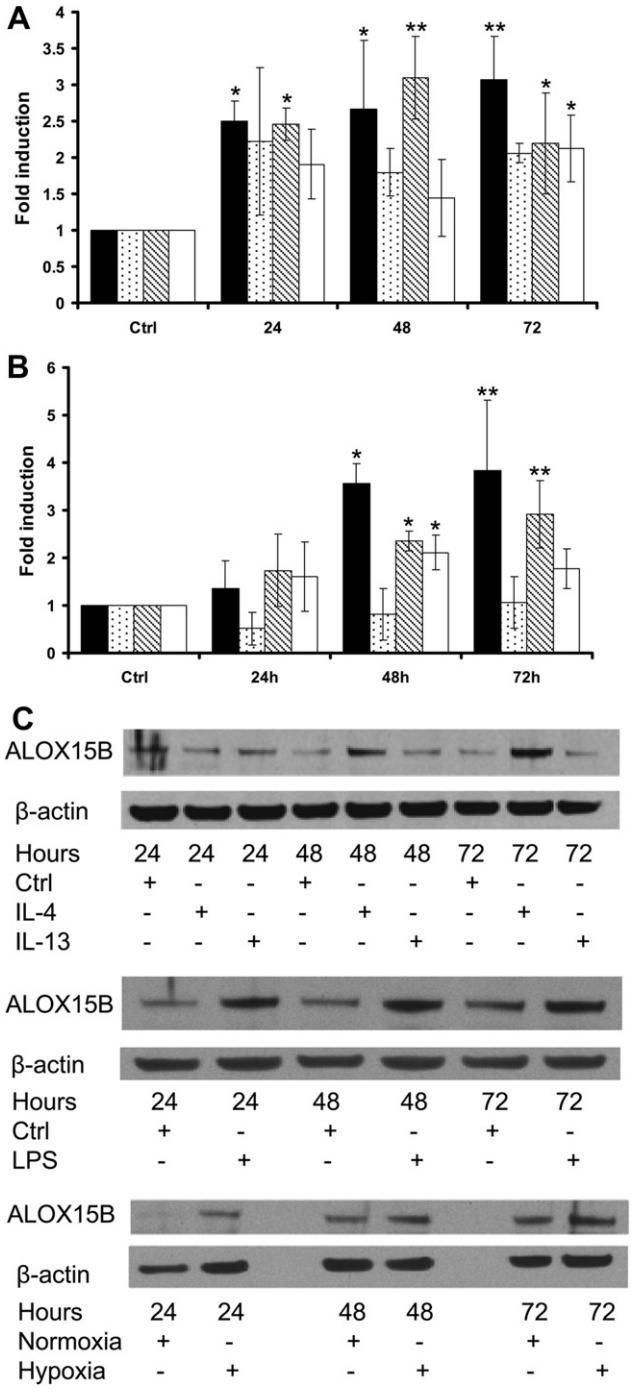

Fig. 5. Expression of ALOX15B after stimulation with cytokines, LPS and hypoxia for 24, 48 and 72 h (IL-4 10 ng/ml, IL-13 10 ng/ml, LPS 100 ng/ml, hypoxic conditions (0.2% O_2, 5% CO_2)). A: mRNA expression following IL-4 (black), IL-13 (dotted), LPS (dashed), and hypoxia (white) stimulation. B: protein expression following IL-4 (black), IL-13 (dotted), LPS (dashed) and hypoxia (white) stimulation. C: representative western blot used for the densitometric analysis following IL-4, IL-13, LPS and hypoxia stimulation. $^{*}p < 0.05$, $^{**}p < 0.01$.

cells [18]. Stimulation of macrophages with INF-γ (released by Th1 cells) and LPS will lead to the classically activated pro-inflammatory M1 macrophages, which secrete IL-1β, IL-15, IL-18, TNF-α and IL-12 [19]. Such M1 cells are characterized by enhanced endocytic functions and enhanced ability to kill intracellular pathogens. In contrast, stimulation of macrophages with IL-4, IL-10 (both released by Th2 cells), IL-13, glucocorticoids and TGF-β will lead to an anti-phlogistic macrophage phenotype M2 involved in tissue regeneration and homeostasis [18,19]. Both types of activated macrophages have been detected in atherosclerotic plaques, although the role of the differently activated macrophages in human atherosclerosis has not been fully elucidated [20,21]. Classically activated M1 macrophages predominate in the lipid core of human carotid atherosclerotic lesions, whereas anti-phlogistic M2 macrophages prevail in the shoulder region as well as in the periphery of the plaque [22]. There is some indication from the secreted cytokine profile of polarized macrophages that classically activated M1 macrophages are pro-atherogenic, while the anti-phlogistic M2 macrophages are atheroprotective [23].

The surprising finding of our study is that human M1 macrophages stimulated by LPS express ALOX15B to high levels. So far 12/15-lipoxygenase activity was only reported for IL-4 stimulated anti-phlogistic M2 macrophages [9] but not for the classically activated M1 macrophages. Our data suggest that such M1 macrophages will specifically express ALOX15B while the M2 macrophages express both ALOX15 and ALO15B isoforms to similar levels. From an enzymatic point of view there are differences between the two 15-lipoxygenases considering substrate preference and positional specificity, which may even influence the role of the two iso-

enzymes in atherosclerosis. In general, ALOX15B seems to preferentially oxygenate arachidonic acid leading to the formation of the anti-inflammatory lipid mediators while it metabolizes linoleic acid to 13(S)-hydro(per)oxy-octadecadienoic acid (13-HpODE) involved in LDL-oxidation to a lesser extend than ALOX15 [24,25]. In addition, ALOX15B was reported to be enzymatically active for longer periods, in contrast to ALOX15 for which rapid suicide inactivation has been observed [25,26]. These substrate preferences and the lack of suicide inactivation may suggest an even more anti-inflammatory metabolite profile for human ALOX15B than for ALOX15.

ALOX15 expression was co-localized with LDL to macrophage-rich areas in early atherosclerotic lesions in humans [27–30] and specific ALOX15 products were observed in these lesions [29,31]. However, conflicting results have been observed about the temporal and regiospecific expression patterns of the peroxidizing ALOX15 enzyme in humans. ALOX15 protein and mRNA were detected in macrophage-rich areas of human fatty streaks as well as in more advanced human atherosclerotic lesions by in situ hybridization [31]. The expression of ALOX15 in early atherosclerotic lesions was corroborated by measuring the specific ALOX15 metabolite 13-HpODE in arterial sections [29,32]. This study showed that the specific ALOX15 metabolite was abundant in early human atherosclerotic lesions but was not in later stages of plaque development, where non-enzymatic lipid peroxidation surpassed the ALOX15 dependent lipid peroxidation [29,32]. Therefore, ALOX15 may play a role in the initiation of atherosclerosis but not in later stages of atherogenesis. However, the expression of ALOX15 in early atherosclerotic development was later questioned by investigations which only detected minor human ALOX15 mRNA and protein in atherosclerotic lesions and found no co-localization of ALOX15 with macrophages [15,33]. Instead, ALOX15B expression was detected in human carotid atherosclerotic plaques and immunohistochemical analysis showed abundant ALOX15B in macrophages-rich areas of carotid lesions [13–15]. In these studies hypoxia was shown to regulate ALOX15B mRNA expression through a HIF-1 alpha mediated mechanism [14]. Our findings corroborate the upregulation of ALOX15B by hypoxia and expand our knowledge on the regulation of ALOX15B in macrophages, showing that the enzyme is not only regulated by HIF-1 alpha but also by IL-4 and LPS. Hence, a larger set of macrophages will have 15-lipoxygenase activity from ALOX15B expression including certain sets of M1 and M2 macrophages.

The preferential expression of ALOX15B in human macrophages may explain why human genetic studies investigating the association of ALOX15 with cardiovascular disease did not show consistent results. Two rare functional polymorphisms have been characterized in the ALOX15 gene, which leads to increased (c. −292C>T) [34] and reduced (T560M) [10] enzyme activity, respectively. While the activating c. −292C>T polymorphism showed a trend towards an atheroprotective effect in a small case–control study for coronary artery disease, the inactivating T560M polymorphism was associated with a significantly increased risk for coronary artery disease in the ADVANCE study, indicating that ALOX15 may be anti-inflammatory and anti-atherogenic in humans [9,10]. However, corroboration of such an atheroprotective effect of the ALOX15 gene failed in a larger case–control study on myocardial infarction possibly because of the low frequency of the T560M polymorphism [11]. So far all large Caucasian study samples investigating the association of the inactivating polymorphism (T560M) in ALOX15 showed a similar risk increase which was, however, not significant in two of the studies [10,11]. In light of our results which emphasize that ALOX15B is the major 12/15-lipoxygenase in human macrophages, further research on the role of the ALOX15B gene in human atherosclerosis would be warranted.

In summary, we show that ALOX15B is the mainly expressed 12/15-lipoxygenase in human macrophages and that its expression is induced by IL-4, LPS and hypoxia. Interestingly, IL-4 and IL-13 also increase the expression of ALOX15 but only IL-4 stimulation seems to drive ALOX15 expression to levels higher than the basal expression of ALOX15B. These expression studies suggest that ALOX15B may play a more important role in human atherosclerosis than ALOX15.

Acknowledgments

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Appendix A. Supplementary material

Supplementary material associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.atherosclerosis.2012.07.022.

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6.3 Personal Contributions to manuscripts

1. **Margot Crucet**, Sophia J.A. Wuest, Patrick Spielmann, Arnold von Eckardstein, Thomas F. Lüscher, Roland H. Wenger, and Christian M. Matter. Differential expression of hypoxia-regulated scavenger receptors modulate lipid uptake in macrophages. (Manuscript I). **All Figures.**

2. Sophia J.A. Wuest, **M. Crucet**, Claudio Gemperle, Angelika Weber, Martin Hersberger Expression of 12/15-lipoxygenases in human primary macrophages. *Atherosclerosis.* July, 2012. (Manuscript II). **Human macrophage isolation for experiments, M1-M2 differentiation and characterization, hypoxia and inflammatory stimuli, protein/RNA extractions.**

3. CA. Schaffner, C. Hiller, **M. Crucet**, GA Kullak-Ublick, JJ Eloranta. Manuscript in Preparation. Regulation of bile acid transporter genes in liver and intestinal cells by hypoxia (Manuscript III-not part of this thesis). **Hypoxia experiments.**

6.3 Unpublished data:

6.3.1Transfection methods tested for RAW 264.7 cells.

RAW264.7 cells are known to be hard to transfect. Several transfection methods were tested to compare their efficiency on RAW264.7 cells.

I.- HiPerFect transfection reagent (Qiagen):

1. Cells were seeded one day before transfection in six well dishes and incubated at a density of approx. 2 x 10^6/mL and grown in DMEM (Sigma). Media were supplemented with 10% fetal calf serum (FCS) and antibiotics (50 IU/ml penicillin and 100 μg/ml streptomycin; Gibco-BRL). Cells were incubated under normal growth conditions (37°C and 5% CO_2).

2. siRNA was diluted to a final concentration of 50nM in culture medium without serum.

3. 40 µl of HiPerFect transfection reagent was added to culture medium without serum, and then added to the diluted siRNA.

4. Samples were incubated for 10 min at room temperature (15–25°C) to allow the formation of transfection complexes.

5. Complexes were added drop-wise onto the cells. Plates were gently swirled to ensure uniform distribution of the transfection complexes.

6. Cells were incubated with the transfection complexes under their normal growth conditions.

7. Culture medium containing serum and antibiotics was changed the following day. Cells were incubated until analysis of gene silencing (24 h after transfection).

II.- Electroporation of Cells (BioRad GenePulserII):

1. Cells were seeded one day before transfection in six well dishes and incubated at a density of approx. 2 x 10^6/mL and grown in DMEM (Sigma). Media were supplemented with 10% fetal calf serum (FCS) and antibiotics (50 IU/ml penicillin and 100 μg/ml streptomycin; Gibco-BRL). Cells were incubated under normal growth conditions (37°C and 5% CO_2).

2. Cells were collected, washed once with PBS, counted, resuspended at 2 to 10 x 10^6 cells/350 μl medium without FCS.

3. Electroporation cuvettes were sterilized with EtOH in the laminar flow work bench

4. siRNA was diluted to a final concentration of 50 nM in culture medium without serum.

5. siRNA and 350 μl of cells were added, gently shaken, electroporated at 1000 μF/250 V/∞Ω (τ at 25 msec) and resuspended in 10 ml medium containing FCS.

7. Culture medium containing serum and antibiotics was changed the following day. Cells were incubated until analysis of gene silencing (24 h after transfection).

III.- Metafectene (Biontex Laboratories):

1. Cells were seeded one day before transfection in six well dishes and incubated at a density of approx. 2 x 10^6/mL and grown in DMEM (Sigma). Media were supplemented with 10% fetal calf serum (FCS) and antibiotics (50 IU/ml penicillin and 100 μg/ml streptomycin; Gibco-BRL). Cells were incubated under normal growth conditions (37°C and 5% CO_2).

2. siRNA was diluted to a final concentration of 50 nM in culture medium without serum.

3. 40 µl of Metafectene transfection reagent was added to culture medium without serum, and then added to the diluted siRNA.

4. Samples were incubated for 20 min at room temperature (15–25°C) to allow the formation of transfection complexes.

5. Complexes were added drop-wise onto the cells. Plates were gently swirled to ensure uniform distribution of the transfection complexes.

6. Cells were incubated with the transfection complexes under their normal growth conditions for.

7. Culture medium containing serum and antibiotics was changed the following day. Cells were incubated until analysis of gene silencing (24 h after transfection).

IV.- Amaxa electroporation (Amaxa Biosystems):

1. Cells were seeded one day before transfection in six well dishes and incubated at a density of approx. 4 x 10^6/mL and grown in DMEM (Sigma). Media were supplemented with 10% fetal calf serum (FCS) and antibiotics (50 IU/ml penicillin and 100 μg/ml streptomycin; Gibco-BRL). Cells were incubated under normal growth conditions (37°C and 5% CO_2).

2. Cells were centrifuged at 90 x g for 10 minutes.

3. After carefully removing the supernatant, the cell pellet was resuspended in 100 μL mouse macrophage nucleofector solution with supplement (Amaxa Biosystems)

4. siRNA (2 μg) or endotoxin-free pmaxGFP plasmid DNA (Amaxa Biosystems) as transfection control was added and the cells electroporated (Amaxa Nucleofector II, program D-032).

5. Immediately afterwards, cells were diluted in 0.2 mL of DMEM and transferred to 10 cm cell culture dishes.

6. Cells were incubated with the transfection complexes under their normal growth conditions.

7. Culture medium containing serum and antibiotics was changed the following day. Cells were incubated until analysis of gene silencing (24 h after transfection).

V.- Lipofectamine (Invitrogen):

1. Cells were seeded one day before transfection in six well dishes and incubated at a density of approx. 2 x 10^6/mL and grown in DMEM (Sigma). Media were supplemented with 10% fetal calf serum (FCS) and antibiotics (50 IU/ml penicillin and 100 μg/ml streptomycin; Gibco-BRL). Cells were incubated under normal growth conditions (37°C and 5% CO_2).

2. siRNA was diluted to a final concentration of 50 nM in OptiMEM®.

3. 40 μl of Lipofectamine transfection reagent was added to 100 μL OptiMEM®,

4. Samples were incubated for 10 minutes, mixed and incubated for 20 min at room temperature (15–25°C) to allow the formation of transfection complexes.

5. Complexes were added drop-wise onto the cells. Plates were gently swirled to ensure uniform distribution of the transfection complexes.

6. Cells were incubated with the transfection complexes under their normal growth conditions.

7. Culture medium containing serum and antibiotics was changed the following day. Cells were incubated until analysis of gene silencing (24 h after transfection).

VI.- Polyethylenimine (PEI)

1. PEI stock solution were prepared: 1 mg/ml PEI in sterile H_2O with 18 μl 1 M HCl/ml, filtered and frozen at -20°C.

2. Cells were seeded one day before transfection in six well dishes and incubated at a density of approx. 2 x 10^6/mL and grown in DMEM (Sigma). Media were supplemented with 10% fetal calf serum (FCS) and antibiotics (50 IU/ml penicillin and 100 μg/ml streptomycin; Gibco-BRL). Cells were incubated under normal growth conditions (37°C and 5% CO_2).

3. siRNA was diluted to a final concentration of 50 nM in culture medium without serum.

4. 40 µl of PEI was added to culture medium without serum and then added to the diluted siRNA.

5. Samples were incubated for 30 min at room temperature (15–25°C) to allow the formation of transfection complexes.

6. Complexes were added drop-wise onto the cells. Plates were gently swirled to ensure uniform distribution of the transfection complexes.

7. Culture medium containing serum and antibiotics was changed the following day. Cells were incubated until analysis of gene silencing (24 h after transfection).

VII.- Lullaby transfection reagent (OZ Biosciences):

1. Cells were seeded one day before transfection in six well dishes and incubated at a density of approx. 2 x 10^6/mL and grown in DMEM (Sigma). Media were supplemented with 10% fetal calf serum (FCS) and antibiotics (50 IU/ml penicillin and 100 µg/ml streptomycin; Gibco-BRL). Cells were incubated under normal growth conditions (37°C and 5% CO_2).

2. siRNA was diluted to a final concentration of 50 nM in culture medium without serum.

3. 40 µl of Lullaby transfection reagent was added to culture medium without serum, and then added to the diluted siRNA.

4. Samples were incubated for 15 min at room temperature (15–25°C) to allow the formation of transfection complexes.

5. Complexes were added drop-wise onto the cells. Plates were gently swirled to ensure uniform distribution of the transfection complexes.

6. Cells were incubated with the transfection complexes under their normal growth conditions for 6 h.

7. Culture medium containing serum and antibiotics was changed the following day. Cells were incubated until analysis of gene silencing (24 h after transfection).

VII. Lentivirus Production using Invitrogen packaging plasmids.

Production of lentivirus-pseudotyped particles

1. HEK 293T cells were plated at a density of 7×10^6 cells per 75 cm^2 culture flask 24 hours before transfection. At least one flask was required per virus.

2. Each plate was transfected with the following transfection mix according to the PEI transfection protocol:

Transfer vector (e.g. pLKO.1, pLenti4 or 6) 3.0 µg

pLP1 4.2 µg
pLP2 2.0 µg
pVSV-G 2.8 µg
total 12.0 µ**g**

3. Medium was replaced the day after transfection with 15 ml fresh media containing serum and antibiotics. Transfection efficiency was visualized by fluorescence microscopy 24 hours post transfection with a pLenti6-EGFP control.

4. Cell culture supernatant was collected 48-72 hours post transfection.

5. Cell culture supernatant was spinned down to remove cell debris, then filtered through a 0.45 µm filter. Viral stocks were stored at 4°C.

Infection using lentivirus-pseudotyped particles

1.Cells were plated the day before infection to reach approximately 50% confluency.

2.A 1:1 mixture of viral stock and fresh media was applied (containing 12 µg/ml polybrene to final concentration of 6 µg/ml).

3.Media was replaced with complete media the day after infection.

4.Selection of the infected cells was started 72 hours after infection using respective antibiotics (puromycin).

6.3.2 Results of transfection tests

Figure 1 shows transfection efficiencies measured by pmaxGFP reporter control (From amaxa kit-lonza) and Western blotting of some of the methods tested.

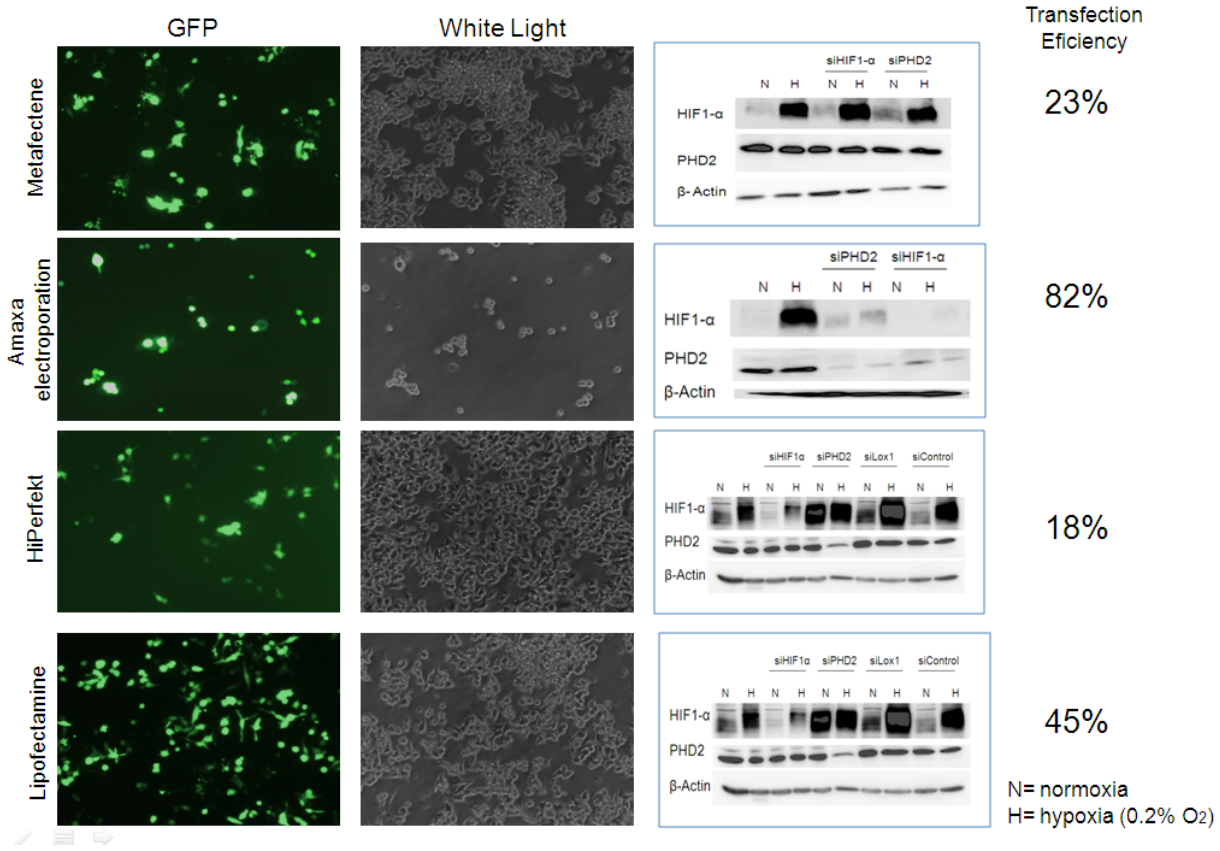

Fig. 1 Metafectene, Amaxa electroporation, HiPerfect and Lipofectamine transfection efficiencies measured by western blotting and fluorescent microscopy.

Amaxa electroporation showed a better transfection efficiency for siRNA against HIF-1α compared with the other methods. We observed a slight decrease in HIF-1α expression levels compared with the siControl. However, HIF-1α levels was still too high.

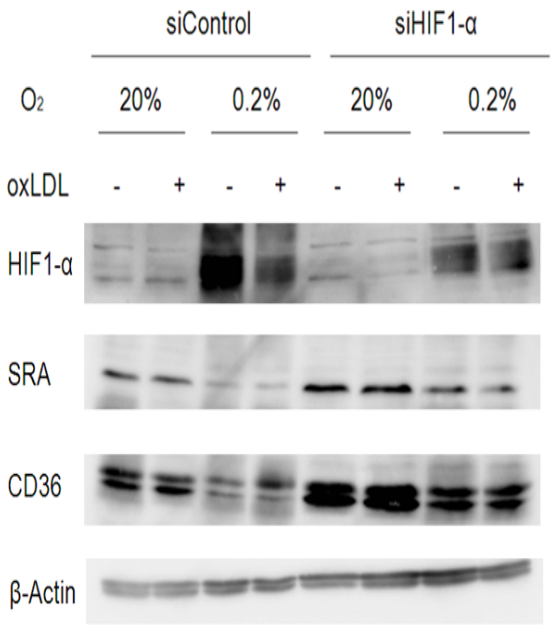

Fig.2 HIF-1α silencing by AMAXA electroporation.

The highest efficiency for transfecting RAW264.7 was obtained by lentiviral infection, as seen by the decrease in HIF-1α expression levels by western blotting (Figure 3).

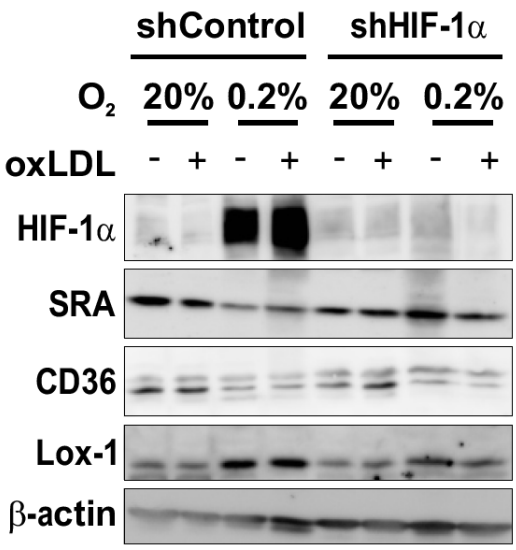

Fig. 3 Immunoblotting of RAW264.7 cells following lentivira transfection of shRNA against HIF-1α.

In conclusion, the best efficient method for transfecting RAW264.7 cells and decreasing HIF-1α levels was obtained using lentiviral approaches with shHIF-1α plasmids. However, we were not able to maintain stable transfections with the selection antibiotic. RAW264.7 cells died two days after infection.

7. Discussion

Atherosclerosis is the leading cause of mortality worldwide. Despite that increasing research is shedding light into the biochemical and cellular mechanisms of atherosclerosis, further studies of the processes that lead to the progression of arterial plaques are still needed for the development of future therapies.

Atherosclerotic plaques contain metabolically active cells whose distances from a blood supply exceed the 100—200-μm diffusion limit for oxygen [1], but still the role of hypoxia in atherosclerosis is not yet fully explored. People exposed to hypoxia in obstructive sleep apnea show increased narrowing of their coronary arteries and increased risk of cardiovascular diseases [2]. Furthermore, chronic intermittent hypoxia has been shown to increase atherogenesis in mice [3].

Hypoxic areas in atherosclerotic plaques have been found in humans and in animal models as small as the rabbit, however the role of hypoxia in mouse atherosclerosis has been controversial. The size of mouse plaques is often within the diffusion distance for oxygen to penetrate from the lumen or the vasa vasorum. Nevertheless, Parathath and coworkers supported the existence of hypoxia in murine plaques by demonstrating the presence of HIF-1α and an increase in the expression of HIF-1α targets. Furthermore, their results showed that hypoxia had a major effect on macrophage lipid metabolism and that HIF-1α promotes changes that would favor foam cell formation [4].

Foam cells, an important hallmark of atherogenesis, are likely to be exposed to hypoxic conditions because of the decreased oxygen diffusion and increased demand in the inner core of the plaque. The lipids in macrophages include cholesteryl esters and triglycerids which are stored in lipid droplets within the cytosol. Their accumulation is mainly due to the uptake of modified lipoproteins, such as oxLDL and acLDL. Hypoxia has been previously reported as responsible for lipid accumulation in macrophages even in the absence of lipoproteins [5]. Furthermore, Glut3 it has recently been proposed a downstream target of HIF-1α for *de novo* lipogenesis in hypoxia-induced lipid loading of human macrophages [6]. Finally, fatty acid synthase is upregulated by hypoxia via Akt and sterol regulatory element binding protein-1 [7].

In this study we showed that both lipid content and oxLDL uptake were increasd following exposure of RAW264.7 to hypoxia. We report a differential modulation of SRA, CD36 and Lox-1 scavenger receptors involved in lipid uptake under this condition, highlighting the role of Lox-1 in atherogenesis.

SRA decreased its mRNA and protein expression following hypoxic exposure in macrophages, while Lox-1 was upregulated at mRNA and protein levels, suggesting a compensatory mechanism under this condition. SRA protein regulation was further studied by the pan-PHD inhibitor DMOG which leads to the stabilization of HIF-1α under normoxic conditions. While HIF-1α protein levels were induced by both hypoxia and DMOG, SRA mRNA and protein levels were reduced in RAW264.7 cells. This hypoxic SRA reduction persisted for 24 hours of 0.5% or 1% oxygen but not beyond: SRA levels partially recovered after 48 hours hypoxia. Since HIF-1α levels were also reduced after 48 hours hypoxia, these data support an involvement of HIF-1α in SRA repression.

Previous studies showed that HIF-1α protein levels were decreased under chronic hypoxia in MCF7 breast cancer cells, while HIF-2α protein levels were increased [8]. The abrogation of SRA decrease under a long exposure time to low oxygen suggests that this receptor can have an atheroprotective role at the early stage of the disease initially reducing foam cell formation. HIF-1α might play a role, either directly or indirectly, in SRA protein reduction under hypoxic conditions. After treatment with different low oxygen concentrations there was, as expected, an increase in HIF-1α protein levels, and a strong decrease in the expression of the SRA protein, suggesting that the effect is not affected by different low oxygen concentrations. $CoCl_2$ and DMOG experiments revealed the involvement of PHD2 in the hypoxic inhibition of the scavenger receptor A, but not in CD36 decrease. Lox-1 increase under hypoxia was clearly affected by DMOG exposure. Further studies are required to investigate the mechanism by which hypoxia is repressing SRA expression and upregulating Lox-1, through a direct or indirect HIF or PHD participation.

Regarding CD36, the contradictory results obtained by western blotting and flow cytometry suggest a relocalization of this receptor following hypoxia. There have been recent publications reporting modulation of scavenger receptors involved in lipid uptake after low oxygen treatments. In line with our results, SRA protein expression levels were shown to be inhibited by 1% of hypoxia and $CoCl_2$ in RAW264.7 cells [9], while CD36 was shown to be increased under low oxygen concentrations in human dermal microvascular endothelial cells, human retinal pigment epithelial and pulmonary artery smooth muscle cells [10]. On the other hand previous studies reported a decrease in CD36 expression following exposure of adipocytes to hypoxia following hypoxia in cardiac myocytes [11], which can explain the differences we observed in CD36 hypoxic regulation with flow cytometry and western blotting.

To the best of our knowledge, our study is the first one to demonstrate the importance of hypoxia induced Lox-1 for lipid uptake in macrophages. Hypoxia-reoxygenation of cardiomyocytes has been reported to induce Lox-1 [12], but there are no other previous studies on the effect of hypoxia on Lox-1 expression. Lox-1, was first identified as a scavenger receptor for binding and uptake of oxLDL in endothelial cells, and it has been shown that Lox-1 plays important roles in pro-inflammatory signaling and atherogenesis [13]. A cross-talk between hypoxia and inflammation has been further supported by the finding that both TNFα and NF-κB upregulate Lox-1 expression [14].

We found Lox-1 to be required for uptake of oxLDL under hypoxic conditions However, hypoxia only slightly stimulated the uptake of acLDL which was not affected by treatment with a blocking anti-Lox-1 antibody. These observations suggest that Lox-1 mediates mainly to the uptake of LDL oxidatively modified by an inflammatory milieu. In fact, we found a co-localization of TNFα and Lox-1 in the inner core of human advanced coronary plaques where the lowest tissue oxygenation could be expected. Bioinformatic analysis identified a putative HRE in the Lox-1 promoter at position -114 (data not shown) which might mediate the HIF effect.

Our study sheds light on the role of Lox-1 as an important player of atherogenesis, increasing lipid content and oxLDL uptake in the hypoxic environment of the advanced plaque. We observed that cells treated with DiL-oxLDL showed increased oxLDL uptake under hypoxia, which was abrogated after exposure of anti-Lox-1 antibody. On the other hand, cells treated with DiL-acLDL showed just a slight increase in acLDL lipid uptake under hypoxia, but there was no difference in cells treated with a blocking Lox-1 antibody (data not shown). These observations suggest that Lox-1 contributes to the overall lipid content and particularly to the increased oxLDL uptake under hypoxia.

Our in vivo results show the presence of TNFα and an increased expression of Lox-1 in the inner core of human advanced coronary plaques where the highest hypoxic microenvironment is expected. Our in vitro data in RAW264.7 cells show that HIF-1α was playing a role in Lox-1 upregulation under hypoxia, and that the blockade of this receptor reduced the hypoxic induction of lipid content and oxLDL uptake under low oxygen concentrations, highlighting its role in advanced atherogenesis. Furthermore, bioinformatic analysis showed the presence of putative hypoxia responsive elements (HREs) in both Lox-1 and SRA promoters at positions -114 and -578 respectively. However further studies are still required to verify the involvement of HIF in the regulation of Lox-1 and SRA under hypoxia.

Macrophages obtained from human atherosclerotic plaques contain mainly cholesterol esters and high amounts of triglycerides. Some reverse cholesterol transport proteins have been reported, including our own work, to be induced in hypoxia. In our screening for differential gene expression involved in oxygen regulated cholesterol transport we observed that mRNA levels from reverse cholesterol transporters such as ABCA1 and ABCG1 were induced under hypoxia. Ugocsai and coworkers demonstrated that HIF-1α binds to the HRE of the ABCA1 promoter increasing ABCA1 activity and expression. They showed that exposure of human primary macrophages to hypoxia or to constitutively active HIF-1α increased ABCA1 expression. Furthermore, they also showed in vivo that in macrophages prepared from human atherosclerotic lesions ABCA1 levels showed a strong correlation with HIF-1β expression. This in vivo

regulatory mechanism was confirmed in human pre-eclamptic placentas, a clinical condition which also presents hypoxia [15]. Moreover, as discussed above, the scavenger receptor CD36 was affected also under hypoxic conditions.

On the other hand, as mentioned bofere, FAS has been also demonstrated to be upregulated under hypoxic environments, as well as SREBP, which is the main regulator of the FAS gene [7]. Moreover, Li and coworkers showed that chronic intermittent hypoxia up regulates genes for lipid biosynthesis in obese mice. They observed the increase in multiple genes controlling 1) cholesterol and fatty acid biosynthesis (malic enzyme and acetyl coenzyme A (CoA) synthetase), 2) fatty acid biosynthesis (acetyl-CoA carboxylase and stearoyl-CoA desaturases 1 and 2); and 3) triglyceride and phospholipid biosynthesis (mitochondrial glycerol-3-phosphate acyltransferase). Furthermore, they demonstrated that majority of overexpressed genes were transcriptionally regulated by SREBP, a master regulator of lipogenesis [3].

Hypoxic environments present at the atherosclerotic lesion may be of importance for the vascular inflammatory processes as well. Using *in vitro* systems, it has been reported that HIF-1α activates NF-kB [16] and that NF-κB controls HIF-1α transcription [17]. Rius and coworkers showed in mice lacking IKK-β, that NF-κB is a major transcriptional activator of HIF-1α and that basal NF-κB activity is required for HIF-1α protein accumulation under hypoxia. The authors performed their experiments in cultured cells and in the liver and brain of hypoxic animals. IKK-β deficiency results in defective induction of HIF-1α target genes including vascular endothelial growth factor. IKK-β is also essential for HIF-1α accumulation in macrophages experiencing a bacterial infection. Hence, this group links IKK-β as an important contributor to the hypoxic response, linking it to both innate immunity and inflammation [18]. Hypoxia induces the expression of a variety of proinflammatory cytokines, such as 15-lipoxygenase-2, eicosanoids, and TNFα [19]. Cytokines induce inflammatory responses including the increased production of lipid droplets [20]. Furthermore, activation of LXR stimulates production of TNFα [21], and hypoxia has been shown to upregulate LXR [22].

Cholesterol rather than triglycerides are the most atherogenic lipids accumulated in foam cells [22]. However, in atherosclerotic lesions, the content of triglycerides is

higher in the musculo-elastic layer and even more in the elastic hyperplastic layer of the intima in comparison to normal aorta, indicating that the increases in triglyceride level also contribute to the pathogenesis of atherosclerosis [23].

Many studies have shown that VLDL particles contribute directly to the intracellular and extracellular accumulation of lipids in atherosclerotic lesions [24]. Low oxygen conditions upregulate VLDL receptors on macrophages [25; 26], and increased levels of VLDL receptors are expressed by plaque macrophages in vivo [26]. These results suggest that hypoxic conditions may further enhance as well the VLDL-associated accumulation of triglyceride in advanced atherosclerotic plaques.

The effect of hypoxia on macrophages showed in this work could play a role in other contexts than atherosclerotic lesions. Tissue and cellular hypoxia is suggested to be of importance in various diseased and inflamed situations such as bacterial infections and wounds, tumors and diabetes. Situations where macrophages are present and accumulate under these circumstances activating gene expression after both, inflammatory and hypoxic stimuli. It is observed that more macrophages are found in ischemic hearts than in control hearts [27]. Moreover, there is an increased formation of lipid droplets and a predominant accumulation of triglycerides in hypoxic human hearts compared with controls [28].

The clinical trials for modulating scavenger receptor function have not been fully successful so far [29]. Since more than a decade ago, adenoviral approaches succeeded to modulate SRA gene expression in vivo [30] however the efficacy was not seen in a long-term basis [29].

The Lox-1 scavenger receptor, as above mentioned, is involved in both early and late atherogenesis. Monitoring and targeting the membrane-bound receptor Lox-1 in vascular tissues or in body fluids could give the opportunity to explore better treatments for vascular and pro-inflammatory diseases like atherosclerosis.

Reports on the impact of available pharmacological therapies on Lox-1 expression in patients are still limited. Morawietz and coworkers demonstrated reduced Lox-1 expression in internal mammary arteries in patients receiving ACE inhibitors [31]. Moreover, clinical trials for endothelial protection, blockage of angiotensin I and statin receptor blockers improved anti-atherosclerotic endothelial expression quotient (which included mRNA expression of vasoprotective genes eNOS and CNP, divided by expression of proatherosclerotic genes Lox-1 and gp91phox) and endothelial function. However, Lox-1 expression itself was not significantly regulated by any medication [32]. Thus, antihypertensive and lipid-lowering drugs currently available may not be sufficient to reduce Lox-1 expression and atherosclerosis in a clinically relevant manner. Even if Lox-1 blocking antibodies have been shown to preserve endothelial function in response to oxLDL in the current study, their application in clinical settings is limited. Further studies towards inhibition of Lox-1 might be an interesting and novel therapeutic strategy in the treatment of atherosclerosis and its clinical implications.

Taking together, I suggest that endogenous content and uptake of lipids overrides the efflux of cholesterol by ABC transporters under hypoxic conditions. Possibly the increased expression of Lox-1 during foam cell formation together with the increased hypoxic signaling in the atherosclerotic lesion may contribute to increases in the levels of cholesterol and triglycerides in macrophages leading to atherogenesis.

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9. Curriculum vitae

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EDUCATION

October 2008 – December 2012

PhD student in Integrative Molecular Medicine

Institute of Physiology, University of Zurich

Thesis title: Modulation of Scavenger Receptors by Hypoxia

Laboratories of Prof. Roland H. Wenger and PD. Dr. Christian M. Matter

September 2005- July 2007

Masters Degree in Clinical Biomedicine

University of Las Americas Puebla, Mexico

General Average: 9.7/10

Thesis Title : Identification of Important Residues of The Origin Binding Protein of Herpes Simplex Virus Type- 1. Perfomed at University of Connecticut Health Center.

September 1999 - June 2004

Bachelors Degree in Biology.

Specialization area: Biotechnology

University of Las Américas Puebla, México.

General average : 9.7/10

Thesis Title: Linguistic Systems compared to Genetic Structures.

January 2000 – July 2004

Bachelors Degree in Hispano-American Literature (Open system)

Specialization area: Linguistics

Centro de Investigación y Docencia en Humanidades del Estado de Morelos, México

General Average: 9.7/10

Thesis Title: Linguistic Systems compared to Genetic Structures.

1996 - 1999

High school

General Average: 9.9/10

Centro Universitario Anglo Mexicano de Morelos (CUAM)

Cuernavaca, Morelos México

EMPLOYMENT:

October 2008 – December 2012

PhD student at Institute of Physiology, University of Zurich

Laboratories of Prof. Dr. Roland Wenger and MD PD Dr. Christian Matter

January 2008 - September 2008

Project Coordinator of Department of Health and Education.

Mexican-American Foundation for Science (FUMEC, Mexico City)

September 2006-June 2007

Graduate Assistant at University of Connecticut Health Center (Farmington, Connecticut)

September 2004 – August 2005

Highschool and Middle School teacher. Comunidad Educativa Caleya (Cuernavaca, Morelos)

Subjects: Biology, Chemistry, Literature and Poetry.

Summer 2002

National University of Mexico (UNAM)

Research assistant in the project:

“p66 gene inhibition with oligonucleotides that form triplex structures”

Laboratory of Dr. Jaime Lagunez

SCHOLARSHIPS AND AWARDS

2007. Magna Cum Laude for Masters Degree. University of las Americas Puebla, Mexico.

2004. Magna Cum Laude for Bachelors Degree. University of las Americas Puebla, Mexico.

2000 – 2004. Decano list award. University of las Americas Puebla: Novus, Juventus, Senatus.

1999. Honorific award for Highschool. CUAM Morelos, Mexico.

October 2008-October 2012

Mexican National Council on Science and Technology (Conacyt) Fellowship.

September 2006-June 2007

University of Connecticut Health Center Fellowship

September 2005-August 2007

Jenkins Scholarship in University of Las Americas Puebla, Mexico (Masters degree tuition fees).

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Mexican National Council on Science and Technology (Conacyt) Fellowship.

September 1999 – June 2004

Jenkins scholarship in UDLAP (Bachelors Degree in Biology tuition fees)

2000 – 2004

Scholarship in Centro de Investigación y Docencia en Humanidades del Estado de Morelos (Bacehelors degree in Literature tuition fees).

1996 – 1999

Scholarship in CUAM Morelos (for tuition fees).

COMPLEMENTARY TEACHING AND POSGRADUATE COURSES AT UNIVERSITY OF ZÜRICH.

Physiology practical courses for medical students and human biology master students at the University of Zurich (230 hours aprox).

LTK course for animal handling. FELASA-category B

Introduction to Human Physiology: Respiration and Blood

Statistics and SPSS

Flow Cytometry

Winterschool 2011: Practical Course in Advanced Microscopy

Project Management for Research.

LANGUAGES:

Spanish , English, German (basic knowledge).

COMMUNICATIONS PRESENTED AT MEETINGS :

- 15th Cardiovascular Research and Clinical Implications Meeting. Murten, Switzerland. October 2009. Poster Presentation.
- 16th Cardiovascular Research and Clinical Implications Meeting. Murten, Switzerland. October 2010. Poster Presentation.
- Zurich Center for Integrative Human Physiology Symposium Meeting. Zürich, Switzerland August 2009. Poster Presentation.
- Zurich Center for Integrative Human Physiology Symposium Meeting. Zürich, Switzerland August 2010. Poster Presentation.
- Zurich Center for Integrative Human Physiology Symposium Meeting. Zürich, Switzerland August 2011. Poster Presentation.
- Swiss Physiological Society Annual Meeting. Fribourg, Switzerland. Oral presentation. September 2010.
- Hypoxia Net Congress. Learning from Hypoxia Signaling. Bilbao, Spain. Oral presentation. April, 2012.

MEMBERSHIPS TO SOCIETIES:

Swiss Physiological Society.

European Atherosclerosis Society.

PUBLICATIONS:

Sophia J.A. Wuest, **Margot Crucet**, Claudio Gemperle, Angelika Weber, Martin Hersberger **Expression of 12/15-lipoxygenases in human primary macrophages.** *Atherosclerosis.* July, 2012. In press.

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LABORATORY TECHNIQUES:

Cellular Biology: Primary cell culture (human monocytes) , RAW264.7 macrophages, Hepa1, Hep3B, HepG2, U2937, Hek293T, U2OS, THP1, Hela, MCF7. Foam cell assays with Oil Red O technique, Flow Cytometry, Fluorescent microscopy, Transient transfections (Amaxa electroporation, Lipofectamine, Metafectane, PEI, etc), stable transfections, lentiviral production and infection (shRNA).

Molecular Biology: RNA and genomic DNA extraction, PCR**,** qRT-PCR, Western blotting, Cloning, Viral Complementation Assays, Promoter studies using reporter Luciferase Assays, Immunoprecipitation.

In vivo: Handling of mice, hypertensive rats (SHRs) and Wistar–Kyoto (WKY) rats.
Histology: immunohisto/cytochemistry, immunofluorescence, confocal imaging .
Bioinformatics: USCS Genome Bioinformatics, Minimotif Miner, Clone manager, Jaspar database.

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